CHARACTERIZATION OF THE REACTION CATALYZED BY ALPHA (2→6) SIALYLTRANSFERASE

By

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This work is dedicated to my parents whom I admire, respect, and love dearly. This dedication is extended to all family and friends who have supported me in this endeavor.

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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF THE REACTION CATALYZED BY ALPHA (2→6) SIALYLTRANASFERASE

By

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Sialyltransferases are glycosyltransferases that catalyze the formation of a glycosidic bond to NeuAc (sialic aid). The resulting sialylated glycoproteins and glycolipids are frequently involved in cell-cell and cell-pathogen binding interactions in addition to other biologically important phenomena. The chemical and catalytic mechanisms of glycosyltransferases have not been well characterized, and this includes sialyltransferases. This dissertation describes mechanistic studies of sialyltransferases, with the long term goal being to use this information for logical inhibitor design.

The first part of this work is a record of the synthesis of the compounds necessary to complete the kinetic experiments. The natural donor substrate for this system, cytidine-5'-monophosphate neuraminic acid (CMP-NeuAc), has been synthesized with a wide variety of isotopic labels located in specific positions. A novel unnatural "slow" donor substrate uridine-5'-monophosphate neuraminic acid (UMP-NeuAc) has also been synthesized containing these isotopic substitutions. The approaches to synthesize, purify, and characterize these molecules are described.

The use of the above mentioned substrates in a variety of kinetic experiments with rat liver $\alpha(2\rightarrow6)$ sialyltransferase is discussed next. These experiments include Lineweaver-Burk analysis, pH-rate profiles, isotope trapping, and inhibitor analysis. The results of these experiments are discussed. Additionally, the results of similar experiments completed using the two different donor substrates (CMP-NeuAc and UMP-NeuAc) are presented.

A variety of kinetic isotope effects have been measured on this system. These effects include β-dideuterium, primary ^{14}C, secondary ^{14}C, ^{18}O leaving group, and solvent isotope effects. These have been measured for the enzyme catalyzed reaction, as well as for the acid catalyzed hydrolysis reactions of the substrates which serves as a model for the enzyme catalyzed reaction. Comparisons are made of the isotope effects measured using the two different donor substrates (CMP-NeuAc and UMP-NeuAc).

The dissertation is concluded with the KIEs measured for another member of the sialyltransferase family, rat liver $\alpha(2\rightarrow3)$ sialyltransferase. The KIE data for the rat liver $\alpha(2\rightarrow6)$ and $\alpha(2\rightarrow3)$ sialyltransferases allow for a comparison of the transition-states of these two enzymes.

CHAPTER 1
INTRODUCTION

Sialyltransferases are enzymes that catalyze the transfer of sialic acids via the activated donor substrate cytidine-5'-monophosphate neuraminic acid (CMP-NeuAc) to an acceptor oligosaccharide (1). Interest in these enzymes arises in part from their role in the regulation of a number of biological processes. The work described in this dissertation represents a study of the chemical behavior of the activated donor substrate CMP-NeuAc as well as the reaction catalyzed by $\alpha(2\rightarrow6)$ sialyltransferase. Work has also been completed on $\alpha(2\rightarrow3)$ sialyltransferase which has allowed for comparisons to be made between enzymes in the same family.

Sialic Acids

In order to appreciate the importance of this class of enzymes, it is first necessary to consider the role of sialic acids. While this introduction does not attempt to be comprehensive on

the subject of sialic acids, it will show how they are involved in a wide array of biological processes. This will underscore how knowledge of the mechanisms of the enzymes controlling sialic acid content could be a first step towards providing a degree of control over a remarkably large number of biological pathways. It is believed that sialic acids function as recognition elements due to their prevalence in peripheral positions on glycoconjugates and in cell membranes (2). An interesting feature of sialic acids is the presence of a highly acidic carboxylate group residing on the anomeric carbon and the role it may play in biological phenomena involving sialic acids. Figure 1-1 illustrates the general structure of free sialic acid which is unstable, and N-acetyl neuraminic acid (NeuAc) which is transferred by sialyltransferase. There are a wide variety of sialic acids which result from modifications of the amino group or hydroxyl groups (2). These compounds are widely distributed in nature with central roles in a number of biological processes (3). Sialic acids are transferred to cell-surface glycoproteins as the "finishing touches" in the synthesis of many biomolecules. Their location

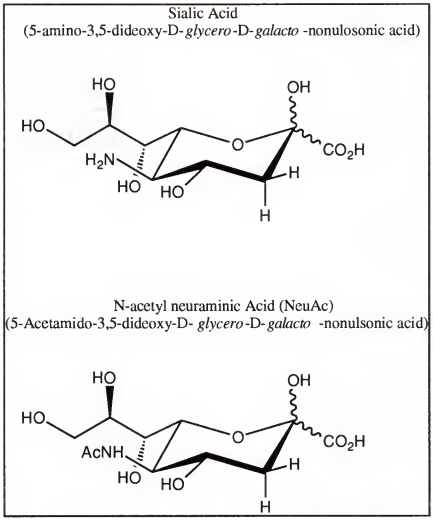


Figure 1-1. Sialic acid general structure and N-acetyl neuraminic acid (NeuAc).

enables them to serve a vital role in cell-cell interactions and regulation. For example, cell-surface sialylated glycoproteins interact with selectins. Selectins are carbohydrate-binding

adhesive proteins (4). The specific interactions of selectins with sialic acid containing molecules is central to the metabolic regulation of many biomolecules (3). A well documented example of this is the interaction of the tetrasaccharide sialyl Lewis x (sLe^x) with various selectins. Sialyl Lewis x has been implicated in inflammatory cell adhesion as well as metastasis of endothelial cells (5, 6). A wide array of studies have been performed to probe the involvement of sialyl residues as selectin ligands (3).

Sialic acids also have functionality in biological processes aside from their roles as recognition elements. Their chemical properties also have biological affects. It has been calculated that $>10^7$ sialic acid residues are bound to the surface of one human-erythrocyte (7, 8). In some instances this electronegative coat can serve to prevent aggregation, as in the case of blood platelets, erythrocytes, and carcinoma cells in culture (9). In other cases the negative charges can act to facilitate aggregation through the use of Ca^{2+} bridges (10). It was also shown that the repulsive, electrostatic forces of sialic acids contribute to the

rigidity of cell surfaces when the treatment of sarcoma cells with sialidase caused an increase in the deformability of the cell (11). The unique influence of sialic acids appears to be mainly due to the negative charges they bear.

Another vital function of these compounds is their influence on the viscosity of biomolecules in the formation of mucous secretions. These mucous secretions act as lubricants and defensive agents at locations of the body that come in contact with the outside environment (12).

One of the most interesting functions of sialic acids is the anti-recognition effect initially demonstrated by Ashwell and Morell. Their studies revealed that sialic acids mask the D-galactosyl residues of serum-glycoproteins thus allowing these molecules to remain in the blood stream. After these sialic acids were enzymatically removed, exposed Gal residues on the glycoproteins were rapidly recognized by D-Gal receptors resulting in the clearance of desialylated molecules from circulation (13). Sialic acids are also involved in the control of mammalian blood cells. Woodruff and Gesner observed that

desialylated lymphocytes are reversibly trapped in the liver and were recirculated in the blood stream after 24 hours. The reappearance was attributed to resialylation of cell membrane glycoproteins in the liver (14). Treatment of erythrocytes from different mammals (including man) with sialidase results in a markedly increased rate of clearance of these cells from the bloodstream (15).

While the presence of sialic acid residues located on the fringes of biomolecules is clearly an important mode of regulation of the lifetime of cells and glycoproteins in the bloodstream, sialic acid has also been implicated in the masking of tumor antigens (16, 17). It has been shown that several chemically induced, malignant tumors of animals can be brought to regression or to disappearance after re-injection of desialylated tumor-cells. Vaccination methods with sialidase-treated tumor-cells have been discussed as possibilities for cancer treatment. These methods hold much promise as more laboratories report that the amount of membrane sialic acids is higher in a variety of tumors than in the corresponding normal

tissues (18, 19, 20). Accordingly, there have been observations of higher sialyltransferase activities in the blood serum from patients suffering from some kinds of cancer. Thus, it seems that some tumor cells can be efficiently masked by sialic acids to avoid immunological attack. Currently, it is reasoned that this hypersialylation, and the resulting decrease of cell-surface D-Gal or GalNAc residues in tumor cells is the reason for the lack of contact with immunological agents allowing the growth of tumor cells (21). An observation supporting this is the decrease in the invasiveness of tumor cells in mouse-heart explants after sialidase treatment (21).

The latest research on the biological roles of sialic acids supports the previous findings and illustrates other effects the degree of sialylation of cells can have. In particular, increased $\alpha(2\rightarrow6)$ sialyltransferase activity has been shown to be correlated with colon cancer. Recent work by Gangopadhyay has shown through the use of monoclonal antibodies (MAbs) that significant increases in the expression of $\alpha(2\rightarrow6)$ sialyltransferase were seen in going from poor to well-developed

colon carcinomas. Very little $\alpha(2\rightarrow6)$ sialyltransferase expression was observed for normal colon tissue (22).

As mentioned, a vital role of sialic acids is involvement in the metabolic clearance of glycosylated proteins, with the trend being that biomolecules remain in circulation longer as their degree of sialylation increases (13). A recent example of this is the prolonged half-life of highly sialylated thyroid-stimulating hormone (23).

While many studies point to hypersialylation as being disruptive of biological processes, there is also evidence that a lack of cell surface sialylation is harmful. The trisaccharide NeuAc-α-(2$\rightarrow$6)-Gal-β-(1$\rightarrow$4)-GlcNAc (sialylLacNAc) is the ligand for the lectin CD22, and is directly related to the regulation of B lymphocyte activation and immune function. It was found that mice which lacked sialylated galactose residues were severely immunosuppressed (24). Additionally, this is seen in cases of carbohydrate-deficient glycoprotein syndromes (CDGS). These are genetic, multi-systemic diseases characterized by deficiencies in the glycosylation of many secretory proteins. A recent study

showed that the cell surface $\alpha(2\rightarrow6)$ sialylation of lymphoblastoid cells from patients with CDGS was much lower than normal (25).

The complexity of sialic acid biology argues that mechanistic study and inhibitor design could yield tools for the study of this area.

Glycosyltransferases and Glycosidases

Glycosyltransferases are a class of enzymes that transfer sugar residues from an activated donor substrate, normally a sugar-nucleotide, to the non-reducing end of an oligosaccharide group (26). The specificity of these enzymes for donor and acceptor substrates and the stereochemistry of the linkages they form determines the structures of the sugar chains produced in a cell. It has been estimated that 100 or more glycosyl-transferases are necessary for the synthesis of the carbohydrate structures on glycoproteins and glycolipids, where most of these are responsible for the diversity of the terminal sequences (27, 28). These biomolecules may be further modified by glycosidases.

Glycosidases catalyze the cleavage of sugar residues with the acceptor nucleophile being water. This class of enzymes is responsible for cleaving or "trimming" carbohydrate residues as needed in various biological processes (29).

A good starting point in the study of the cleavage and transfer of sialic acids is the acid catalyzed hydrolysis of acetals. This is shown in Figure 1-2 (30). In this example, there

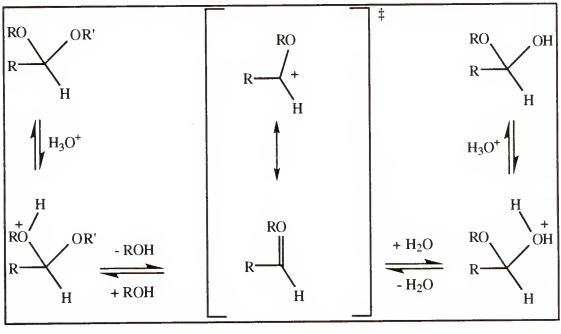


Figure 1-2. A general scheme for specific acid-catalyzed acetal hydrolysis.

is specific acid catalysis to cleave the leaving group bond. Enzymatic reactions may possess some of the same characteristics of these reactions, such as the utilization of an acid catalyst for glycosidic cleavage, and a transition-state with oxocarbenium ion character.

It is also worthwhile to review the catalytic mechanism of hen egg white lysozyme (HEWL), the paradigmal retaining glycosylase. While the nature of intermediates along this reaction pathway is still unresolved, the known features may share similarities to glycosyltransferases. The reaction is illustrated in Figure 1-3. The Phillips hypothesis proposes the formation of an enzyme bound oxocarbenium ion after cleavage of the glycosidic bond which is ion-paired with the carboxylate of Asp 52 (upper branch of Figure 1-3) (31). Alternatively, it has been proposed by Koshland that Asp has a nucleophilic role in catalysis (lower branch of Figure 1-3) (32). These are possibilities that should be kept in mind when studying glycosyltransfer reactions.

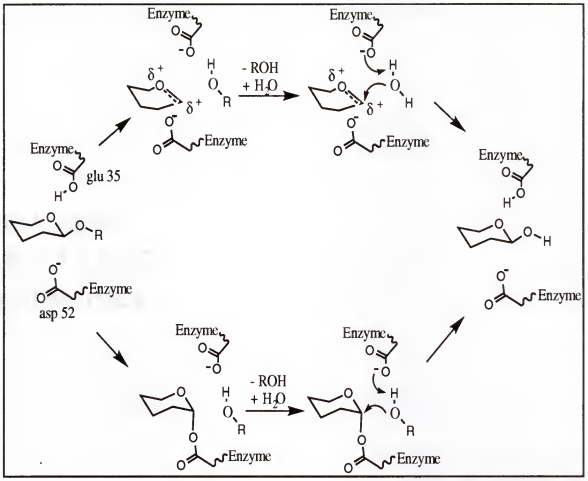


Figure 1-3. Lysozyme mechanism with respect to active site Asp and Glu carboxyls.

While glycosyltransferases are not as well studied as glycosidases, the mechanism of α-1,3-fucosyltransferase V has been explored and may share similarities with other

glycosyltransferases. This enzyme transfers 1-fucose via the donor sugar guanosine-5'-diphospho-β-1-fucose (GDP-Fuc) to an acceptor sugar. Similar to sialyltransferase, this reaction proceeds with inversion of configuration at the anomeric center (33). Through the use of α-secondary isotope effects, a mechanism was proposed for α-1,3-fucosyltransferase V in which cleavage of the glycosidic bond occurs prior to nucleophilic attack at the anomeric carbon, as shown in Figure 1-4 (34). Evidence for a general base catalyzed mechanism (presumably a carboxylate) was seen in the pH-rate profile by the identification of a catalytic group with an apparent pK_a of 4.1. Another interesting feature of the α-1,3-fucosyltransferase V mechanism is that the enzyme requires a metal co-factor. The enzyme performs optimally with Mn^{2+}, but it can function with other divalent metals (34). The presence of the carboxylate group adjacent to the anomeric carbon for the sialyltransferase substrate (CMP-NeuAc) will certainly have an impact on its

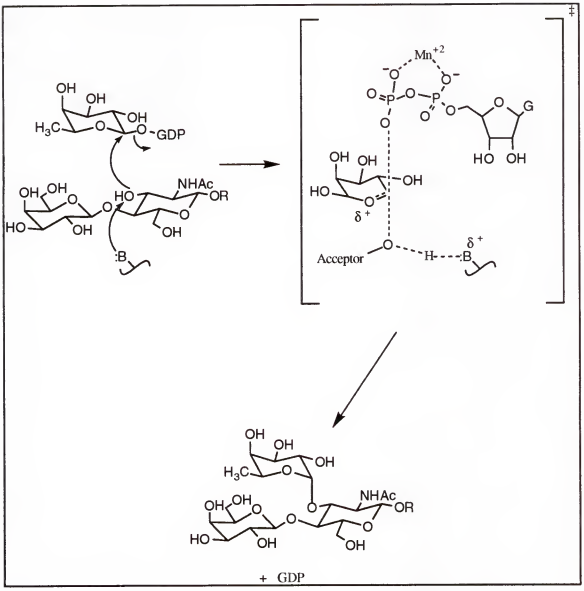


Figure 1-4. Proposed mechanism of α-1,3-fucosyltransferase V (Taken from reference 34).

reactivity, but the reaction mechanisms for sialyltransferases and other glycosyltransferases may share similar features.

Sialidase and Trans-sialidase

The glycosyltransferases and glycosidases we are interested in are those which act upon sialic acids. These are the sialyltransferases and sialidases. Sialidases, or neuraminidases, transfer carbohydrate-linked sialic acid to water. Some trypanosomes have trans-sialidases which transfer carbohydrate-linked sialic acids to other carbohydrates (35). Trans-sialidases are unusual glycosyltransferases because they do not use sugar-nucleotides as glycosyl donors. The reactions catalyzed by these enzymes are illustrated in Figure 1-5.

The trans-sialidase is an interesting enzyme that is able to catalyze both the hydrolysis and transfer of sialic acids. This enzyme is expressed on the surface of *Trypanosoma cruzi*, the agent of Chagas disease, and catalyzes the transfer of $\alpha(2\rightarrow3)$-linked sialic acid to acceptors containing terminal β-galactosyl residues. The parasite uses this enzyme to acquire sialic acids from the host in order to provide a defense against the host's immune system. The trans-sialidase also has some hydrolase ability (36).

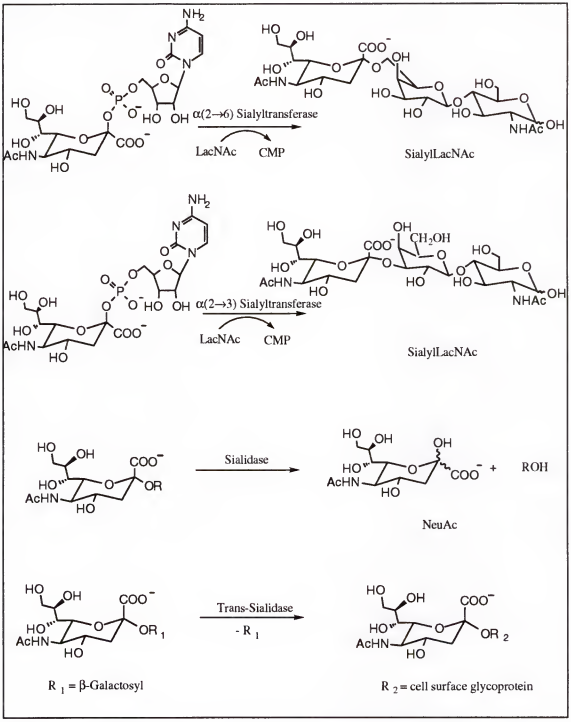


Figure 1-5. Reactions of α(2→6) sialyltransferase, α(2→3, sialyltransferase, sialidase, and trans-sialidase.

The mechanism of the enzymatic and chemical hydrolysis of glycosides has been well characterized and is a good starting point in the study of glycosyltransferases. The mechanism of the hydrolysis of NeuAc aryl glycosides has been investigated by Sinnott. In this work, it was demonstrated that the transition-state had significant oxocarbenium-ion character and required some nucleophilic assistance from the C1 carboxylate group (37). This has implications for the catalytic cleavage of sialic acid by sialidases, enzymes that cleave terminal NeuAc residues found in oligosaccharides. A mechanism for the influenza sialidase has been proposed in which there is no enzymic nucleophile approaching the β-face of the sugar. Instead, Glu 277 is positioned on the α-face to stabilize the oxocarbenium ion electrostatically. In this case it is possible that the carboxylate group is acting as the nucleophile (38).

In animals, sialidases are located in several types of tissues and are involved in the regulation of the surface sialic acid profile of cells (39). The interplay of sialyltransferases and sialidases may help to finely tune the biological processes

previously discussed. Part of the interest in sialidases has arisen because of their involvement in a number of diseases as virulence factors. Sialidases have been implicated as virulence factors for viruses, bacteria, and parasites (37). For example, neuraminidases work in conjunction with hemagglutinin (HA) during the life cycle of influenza virus. HA mediates target cell recognition by specifically binding to cell surface receptors bearing sialic acids and is also involved in the fusion of viral and cell membranes. Neuraminidases catalyze the hydrolytic cleavage of cell surface sialic acids which is thought to aid in the transport of the virus by facilitating passage through the mucin and preventing self-aggregation (40).

Sialidase Inhibitor Design

The structure-based design of potent inhibitors of the influenza-virus neuraminidase was a great success of rational drug design, and it is not unreasonable to suspect that the catalytic mechanism of sialidases could be similar to that of sialyltransferases. The fact that sialidases have been well

characterized makes them a reasonable starting point in studies attempting to understand the catalytic mechanism of sialyltransferases.

The influenza-virus neuraminidase is a homotetramer with a monomer molecular weight of ≈42 kDa (41). Its three dimensional structure was determined in 1983 (42). Sequence identity between the influenza enzymes and the bacterial enzymes is low (15%), but there is structural and active site conservation (43). The active site structures of sialidases were elucidated through complexes with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) (Figure 1-6) which inhibits most sialidases with a $K_i=10^{-6}$ M (44). This compound is considered to be a transition-state analog, by virtue of its flattened ring serving as a mimic for the proposed oxocarbenium-ion formation and planarization of the NeuAc ring thought to occur at the transition-state (45).

N-acetyl-neuraminic acid (Neu5Ac) is a weak inhibitor of the enzyme with $K_i=10^{-3}$ M. Further analysis of the active-site of

the influenza enzyme revealed the presence of two glutamic acid residues located in a pocket around O4 of the sialic acid ring.

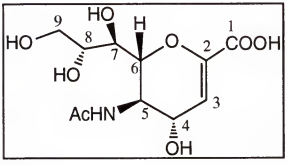


Figure 1-6. Neu5Ac2en.

These residues were also found to be conserved across all known strains of influenza A and B viruses. This observation led to the design and synthesis by Glaxo of a compound with the O4 group of Neu5Ac2en substituted with a guanidinium group. This compound, named GG167, is a potent inhibitor of the influenza enzyme with $K_i=10^{-11}$ (46). It is interesting that GG167 is specific for the influenza enzyme and is a poorer inhibitor of nonviral sialidases (47). It has also been shown that this inhibitor has a slow on-rate which has been attributed to the need to remove a water from the active-site (48).

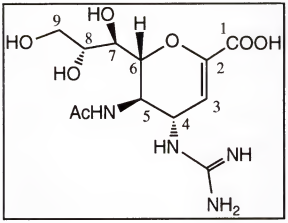


Figure 1-7. GG167.

With GG167 as a lead compound, other inhibitors have been designed to probe the active-site for favorable interactions. This work has identified the importance of the N-acetyl and glycerol moieties in binding (49, 50). The design of GG167 was, in part, made possible due to the elucidation of the active-site architecture of the influenza sialidase from the crystal structure (39). While crystal structures are currently unavailable for sialyltransferases, the information obtained on sialidases should be kept in mind given the similarities between the substrates and reactions catalyzed by the two classes of enzymes.

Sialyltransferase

Sialyltransferases catalyze the transfer of N-acetyl neuraminic acid (NeuAc) via the activated donor substrate cytidine-5'-monophosphate N-acetyl neuraminic acid (CMP-NeuAc) to an oligosaccharide acceptor (1). There are at least 15 enzymes in this group (24). Currently, cDNA clones have been obtained for about 15 animal and bacterial sialyltransferases (51, 52). These enzymes are classified by their acceptor substrate specificity. The acceptor oligosaccharide may be one of a variety of oligosaccharides with a terminal galactose residue or even another NeuAc residue. Additionally, there may be a requirement for the terminal galactose to be acylated. Each sialyltransferase transfers NeuAc to a hydroxyl group of the β-galactoside acceptor. All sialyltransferases catalyze the formation of a glycosidic bond linked to C2 of NeuAc in the α conformation (53), but differ in their specificity for the acceptor hydroxyl group. Sialyltransferases that transfer NeuAc to the C2, C4, C6, and C8 hydroxyl groups have been identified. As an

example, the Galβ(1→4)GlcNAc α(2→6) sialyltransferase is responsible for the production of the NeuAc(2→6)Galβ(1→4)-GlcNAc linkage. This represents the transfer of NeuAc to the C6 hydroxyl group of LacNAc (Gal-GlcNAc) with the glycosidic bond being in the α configuration (54).

Protein sequence analysis of sialyltransferases has shown that they share gross structural features common to other glycosyltransferases. This is consistent with their function as enzymes involved in the post-translational glycosylation of glycoproteins. Each has a short amino-terminal cytoplasmic domain, a hydrophobic signal-anchor sequence functioning as the membrane-spanning domain, a lumenal "stem" region (not catalytically required), and a large lumenal catalytic domain (55).

Apart from these common topological features there is no sequence homology between sialyltransferases and other glycosyltransferases, nor is there any sequence homology with glycosylases, including neuraminidase. However, there is some sequence homology between sialyltransferases: 10-12% of a

region in the middle of the catalytic domain, and a smaller sequence consisting of 23 residues at the carboxy terminus of the

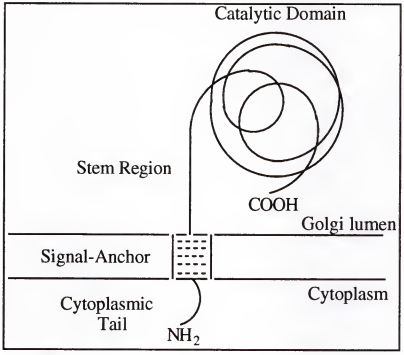


Figure 1-8. Common topology of cloned terminal glycosyltransferases (Taken from reference 44).

sequence (56). The largest conserved region, called the L-sialylmotif is a region of 48 or 49 amino acids which exhibits 40-60% homology between any two sialyltransferases and contains eight invariant residues in all the cDNAs cloned to date

(57). The L-sialylmotif is a region of about 23 amino acids with two invariant amino acids (58). It was presumed that the sialylmotifs contributed to the common function of the sialyltransferases, namely the transfer of sialic acids via the activated donor substrate CMP-NeuAc.

To investigate the function of the L-sialylmotif, a site-directed mutagenesis study was done on the rat liver $\alpha(2\rightarrow6)$ sialyltransferase. Eleven mutant enzymes were constructed in which selected conserved amino acids were changed to alanine. Most of the mutants retained sialyltransferase activity and had increases in the K_m of CMP-NeuAc without corresponding changes in the K_m of the acceptor substrate (55). These results suggest that the L-sialylmotif of the $\alpha(2\rightarrow6)$ enzyme is involved in the binding of the donor substrate, CMP-NeuAc. Similarly, an analysis of the catalytic activity of sialyltransferases with mutations in the S-sialylmotif was done. In this study a total of 9 mutants were prepared with wild-type residues changed to alanine. Kinetic analysis of catalytically active mutants revealed that mutations in the S-sialylmotif resulted in a change in K_m for both

substrates. In most cases K_m values increased suggesting that this region is involved in the binding of both the donor and acceptor substrates (58). Three dimensional crystal structures of sialyltransferases have not been reported. This may be due in part to the difficulty in their purification and that recombinant sialyltransferases are not readily obtained from bacterial expression systems.

Sialyltransferase Inhibitors

Until recently, a systematic search of sialyltransferase inhibitors had not been reported. In the 1970's the influence of nucleotides on the sialyltransferase activities of rat liver microsomes was investigated (59, 60), but the inhibition of purified sialyltransferases was not well documented until 1997 by Schauer and co-workers (61). Studying purified enzymes is important because inhibitors may influence the enzyme's apparent activity by having an effect somewhere else along the overall pathway of a biological process. In Schauer's study, the inhibition of $\alpha(2\rightarrow6)$ sialyltransferase from rat liver and $\alpha(2\rightarrow3)$

sialyltransferase from porcine submandibular gland were studied using a series of different sialic acids, nucleosides, nucleotides and nucleoside conjugates with sialic acid or galactose. The array of inhibitors tested were chosen to attempt to identify the structural elements important for inhibition. Very little inhibition was observed for any sialic acid derivatives. In contrast to this result, some of the nucleosides and nucleotides showed significant inhibition. The most effective inhibitors of $\alpha(2\rightarrow6)$ sialyltransferase were the cytosine derivatives with the inhibition increasing in the following order: 5'-CMP (K_i=90μM), 5'-CDP (K_i=50μM), 5'-CTP (K_i=46μM). The increase in inhibitory activity by adding phosphate groups may be due to their ability to mimic the negative charge of the carboxylate group of CMP-NeuAc. Cytosine and ribose showed no inhibition indicating that the nucleoside monophosphate is the minimal unit necessary for inhibition (61).

Recently, a synthesis of sialyltransferase inhibitors was carried out by Schmidt and co-workers. Their strategy was to attach the CMP moiety to the tertiary hydroxyl group of quinic

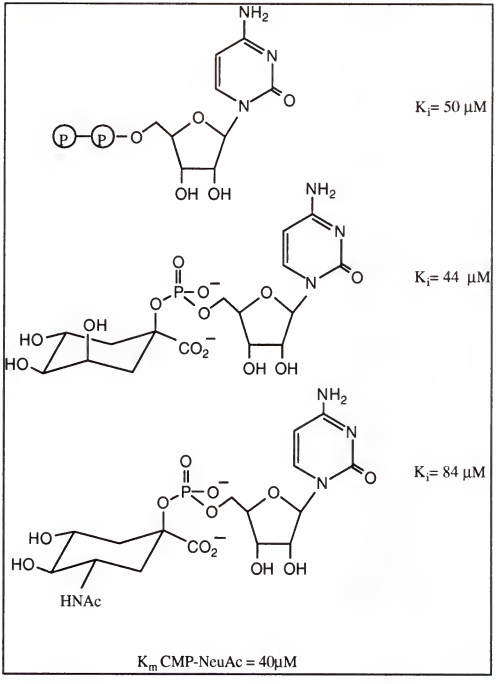


Figure 1-9. CDP and quinic acid based sialyltranferase inhibitors (Taken from reference 62).

acid. The qualities that make these good lead compounds as sialyltransferase inhibitors are the presence of the functionalities associated with CMP, the replacement of the substrate glycosidic-bond with a C-glycoside linkage making it chemically inert, and presumably high stability under physiological conditions. The K_i of 44 μM for the best inhibitor in this group is an improvement over previous sialyltransferase inhibitors (62), but is in the same range as the value of K_m for CMP-NeuAc. With the observation that the CMP moiety was very important in binding, Schmidt and co-workers employed a new strategy in which transition-state geometrical mimics were attached to a CMP group as shown in Figure 1-10 (63). This molecule consists of 2,3-dehydro-NeuAc linked to CMP with a phosphonate group attached to the linkage. It was envisioned that the phosphonate group could mimic the carboxylate functionality of NeuAc. The hypothesis that the sialyltransferase proceeds via a transition-state which possesses substantial oxocarbenium ion character calls for the transition-state geometry to be that of a flattened ring, which the dehydro NeuAc should mimic. The K_i of

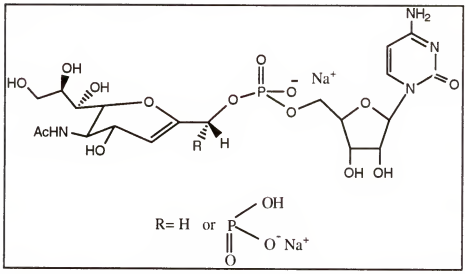


Figure 1-10. Transition-state geometrical mimic tethered to CMP (Taken from reference 63).

the phosphonate compound illustrated in Figure 1-10 was determined to be 0.35 ± 0.5 μM. This compound is bound by the enzyme more than 100 times as tightly as substrate (CMP-NeuAc) and is the first sialyltransferase inhibitor to be bound more tightly than substrate.

Schmidt then checked to see if the functionality of the neuraminyl ring was important, or if the flattened geometry was giving the compound its inhibitory properties, by synthesizing and testing the inhibitors shown in Figure 1-11. These compounds

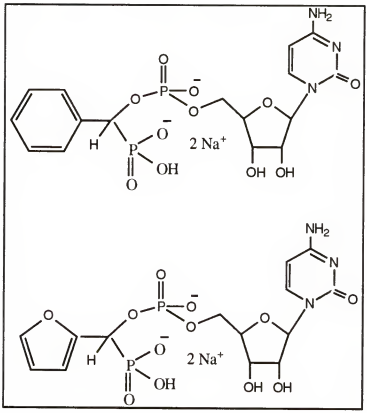


Figure 1-11. Benzene and furan tethered to CMP moiety (Taken from reference 63).

replace the neuraminyl group with a benzene or furan group. The K_i for the benzyl compound was 0.20 ± 0.5 μM, and the K_i for the furanyl compound was 0.28 ± 0.6 μM. These results suggest that the functionality of the neuraminyl group is not extremely important and that the inhibitory properties are due to the

flattened hydrocarbon ring. However, if the phosphonate group is changed to a carboxylate, the binding affinities of these compounds are decreased by an order of magnitude. Additionally, the configuration at the center isoteric with the anomeric carbon of NeuAc does not affect the inhibition constants. This information was then used to design an inhibitor which possessed a flattened ring as well as a flattened linkage to CMP and a phosphonate group. This inhibitor is shown below in Figure 1-12.

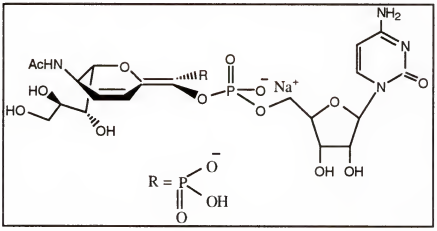


Figure 1-12. Sialyltransferase inhibitor possessing a pyranosyl ring mimic and a phosphonate bearing methylene (Taken from reference 63).

The K_i for this compound is 0.04 ± 0.08 μM which represents a binding affinity 1000 times greater that that of the natural donor substrate CMP-NeuAc.

This class of sialyltransferase inhibitors represents an important advance in this area of research. For the first time there is a class of inhibitors which are bound by the enzyme more tightly than substrate. The tethering of transition-state geometry mimics to CMP has a synergistic effect which is drastically increasing the binding affinity of the compounds.

Information with respect to the transition-state geometry may be obtained by the measurement of kinetic isotope effects (KIEs). These experiments are capable of giving detailed information on transition-state structure, thus being an ideal approach for obtaining mechanistic information as well as inhibitor design. X-ray crystallographic data for the sialyltransferase active site would be extremely useful in the design of effective inhibitors, however, this information is currently unavailable. In the case where crystal structures are not available, it is necessary to use alternative means to gain

insight into the active site structure and mode of catalysis for a given enzyme. Additionally, even when crystal structures are available, they don't give information about the transition-state of the reaction.

Kinetic Isotope Effect Background

The basic idea behind an isotope effect is relatively simple. It is the relative rate of two molecules which differ only in isotopic substitution at one position in the molecule. Conversely, the origin and interpretation of isotope effects can be very complex. Conventionally, isotope effects are expressed as the rate of the light molecule over that of the heavy.

The use of isotope effects to probe chemical reaction mechanisms was in great part made possible by the work of Bigeleisen and Mayer (64). Their initial paper "Calculation of Equilibrium Constants for Isotopic Exchange Reactions" published in 1947 laid the groundwork for the field of isotope effects. Initially, equilibrium isotope effects were addressed and later extended to isotope effects on reaction rates. The Bigeleisen

equation for equilibrium isotope effects is given in equation 1.1. In this equation K_1 and K_2 are the equilibrium constants for the two isotopic species being measured ZPE represents the contribution resulting from the effect of zero-point energy differences between the initial and final state

$$K_1/K_2 = MMI * EXC * ZPE \qquad \text{eq. 1.1}$$

of the two isotopic species. The term EXC is the effect if conditions exist in which vibrational energy levels above the zero level are populated. Finally, MMI represents the combined molecular-mass and moments of inertia (65). The parameters that constitute the individual terms in eq. 1.1 are listed below.

Isotope effects on rates are also represented by the Bigeleisen equation in almost the same form. The normal-mode frequencies are treated in a slightly different way because transition-state theory requires that one normal-mode have a zero or imaginary frequency, $\nu^{\ddagger}_{L}$. This mode is referred to as the reaction coordinate mode because motion along this coordinate is

the transformation of the transition-state back to reactants or forward to products. Therefore, transition-states have 3N-7 frequencies and one imaginary frequency, while ground-states have 3N-6 frequencies (64).

$$MMI = \left(\frac{M_1^{\ddagger}}{M_2^{\ddagger}} \times \frac{M_2}{M_1}\right)^{3/2} \left(\frac{A_1^{\ddagger} B_1^{\ddagger} C_1^{\ddagger}}{A_2^{\ddagger} B_2^{\ddagger} C_2^{\ddagger}} \times \frac{A_2 B_2 C_2}{A_1 B_1 C_1}\right)^{1/2}$$

$$EXC = \prod_{i}^{3N-6} \frac{1-e^{-u_{i(1)}}}{1-e^{-u_{i(2)}}}$$

$$ZPE = \prod_{i}^{3N-6} \frac{e^{(1/2)\,u_{i(1)}}}{e^{(1/2)\,u_{i(2)}}}$$

Figure 1-13. Expanded terms of the Bigeleisen equation.

Isotope effects are generally dominated by the ZPE term with most of the isotope effect coming from the difference in the changes in zero point energy either between reactant and product or in going from the ground-state to the transition-state.

The magnitude of the zero point energy factor for the isotope effect on rate is dependant upon the change in force constants to the isotopically substituted atom upon going from the ground-state to the transition-state. Typically, if there is no change in bonding at the point of isotopic substitution this term will be unity. If the bonding decreases (i.e., the isotopically substituted atom is in a looser environment in the transition-state than in the ground-state), this term will be greater than unity, usually resulting in a normal (>1) isotope effect. Qualitatively this means the light isotope reacts faster than the heavy isotope. If the bonding to the isotopically atom increases (i.e., tighter bonding environment in the transition-state), the zero point energy factor will be less than unity resulting in an inverse (<1) isotope effect. This can be summarized by the first law of isotope chemistry: the light isotopic molecule prefers the state in which the restrictions to vibration are lower, i.e., the state in which the bonding to the isotopic atom is "less stiff" (66).

While the basis of all kinetic isotope effects are a change in force constants between the ground-state and transition-

state, there are different types of isotope effects with different origins. The two major classes are primary and secondary effects. A primary isotope effect occurs when the bond to the isotopically substituted atom is broken, whereas a secondary isotope effect arises when the bond to the isotopically substituted atom remains intact, but experiences a change in its bonding environment during the reaction. Secondary isotope effects can be further divided into alpha and beta effects. A secondary alpha isotope effect occurs when the isotopically substituted atom is bonded to the atom where the bond is being broken or formed. A secondary beta effect occurs when the isotopically substituted atom is not attached directly to the atom where bond cleavage or formation is occurring, but is one atom removed from the reaction site (67). The different classes of isotope effects as well as their origins and interpretations will be discussed later in more detail.

Background on Isotope Effect Measurement Technique

When measuring kinetic isotope effects, the experimental protocol is of the utmost importance. Many of the most informative effects are very small (as low as 1%) and thus require an experimental technique that is capable of delivering accurate and precise numbers. The experimental approach used to measure the effect will also determine whether the effect can be measured on V/K, V_{max} or both. Isotope effects on enzymatic reactions may be measured either competitively or noncompetitively depending on the type of effect one desires to measure. A discussion of experimental designs to measure kinetic isotope effects on enzyme catalyzed reactions and their caveats follows.

Competitive Method

The competitive method involves using a mixture of two labeled substrates which are exposed to the enzyme simultaneously. Two frequently used isotopes for this approach

are ^{3}H and ^{14}C. Usually the substrate pair will consist of one with a radiolabel at the isotopically sensitive position, while the other contains a radiolabel at a remote position. The remote label will report on the naturally occurring isotope at the isotopically sensitive position. Another variation of this method is the combination of stable isotopes with radiolabels. In this case the stable isotope is synthesized into the isotopically sensitive position and a radioactive label is synthesized into a remote position on the molecule. The competitive method is attractive because of the small experimental errors which are obtainable. For example, $^{3}H/^{14}C$ ratios can be determined to a precision of 0.3%. In cases where stable labels are used precisions of 0.001% are easily obtained if a gaseous product is available for measurement by gas ratio mass spectrometry (68). However, a disadvantage is the need for significant amounts of radiolabeled substrates. They can often be difficult to synthesize and require expensive radioactive precursors. This requirement does not exist for gas-ratio mass spectrometry which utilizes natural isotopic abundances and is the most accurate technique

for small isotope effects. However, the number of reactions that this technique is suitable for is limited because of the need for the isotopically sensitive portion of the molecule to be converted to a gas. This conversion must also proceed without an isotope effect (68). A general limitation of the competitive method is that it only yields V/K effects (69, 70). While the reason for this may not be obvious initially, it lies in the fact that the two substrate isotopomers can never both be saturating since they can be considered competitive inhibitors of each other.

When using remote labels for the competitive method, it is necessary to demonstrate that the label is in an isotopically insensitive position. This control can be done by using substrates that each have the radiolabel in the remote position and measuring the KIE. A KIE of unity will be observed it the remote positions do not have an isotope effect associated with them. It is not uncommon for remote ^{3}H labels to exhibit small KIEs resulting from binding on the enzyme (71, 72). If the KIEs associated with the remote label are accurately known, the

observed effects on other positions can be corrected to take this into account.

A control must also be performed to ensure that there is no isotope effect associated with the method itself. Specifically, measurement of the isotope effect by competitive methods requires separation of products from reactants. For example, if the substrate or product is being purified chromatographically, this purification step must be shown to be isotopically insensitive. Purification techniques can introduce isotope effects if proper care is not taken (73, 74). This situation can usually be avoided by collecting the product or substrate in its entirety so that if an isotopic separation does occur upon purification, it will not matter because everything was collected.

The final issue to be addressed concerning the dual label method is the liquid scintillation counting of the samples. This is accomplished in a multi-channel liquid scintillation counter in which one channel (A) detects counts arising from ^{3}H and ^{14}C, while a second channel (B) detects only those arising from ^{14}C. The amount of ^{3}H and ^{14}C in an unknown mixture can be calculated

through the use of a ^{14}C standard which indicates the ratio of ^{14}C in channel A and channel B. The approach is shown below. The $^{3}H/^{14}C$ ratio can then be calculated. The observed isotope effect is how this ratio changes upon running the reaction (67).

^{14}C CPM = CPM channel B + (CPM channel B * A/B ^{14}C ratio) eq. 1.5

^{3}H CPM = CPM channel A - (CPM channel B * A/B ^{14}C ratio) eq. 1.6

Our method measures the change in the isotopic ratio from 0% conversion (t_0) to somewhere close to 50% conversion ($t_{1/2}$). Duggleby and Northrop have demonstrated that the most accurate results are obtained from experiments reaching 50% conversion. They showed that errors increase significantly near 0 and 100% conversion (75). The error on the KIE was also shown to be correlated with the magnitude of the effect. Errors are greatest for small KIEs when measuring ratios of products, whereas they are greatest for large KIEs when measuring ratios of remaining reactants (75). The observed isotope effect is given by eq. 1.7

(69). Because isotope effects are written as the rate of the light over that of the heavy, it is assumed in this equation that ^{3}H is

$$KIE_{obs} = (^{3}H/^{14}C)t_0 / (^{3}H/^{14}C)t_{1/2} \qquad \text{eq. 1.7}$$

reporting on the light isotope and ^{14}C on the heavy isotope. For the case in which ^{14}C is reporting on the light isotope, the numerator and denominator should be inverted. Due to the fact that the isotopic composition of the reaction mixture is constantly changing, the observed isotope effect must be corrected for the fraction of conversion (f) of the substrate to product as shown below in eq. 1.8 (76). This equation by Bigeleisen and Wolfsberg adjusts the KIE back to 0% conversion.

$$KIE_{corr} = \ln(1-f*KIE_{obs}) / \ln(1-f) \qquad \text{eq. 1.8}$$

When performing an experiment, the reaction is usually run in triplicate to give three t_0's and three $t_{1/2}$'s. These are then

counted for a minimum of 6 cycles through the liquid scintillation counter with each tube being counted for 10 minutes. In order to allow for good counting statistics, it is suggested that the sample to be counted should contain at least 100,000 CPM. This will reduce the relative error in counting from cycle to cycle. Each sample should be identical with respect to buffer composition as well as volume and amount of scintillation fluid added. These variables can influence the amount of quenching and the channel A/B ratio (68).

Non-competitive Method

The direct comparison of reaction rates of labeled and natural-abundance substrates in separate reactions is performed in the non-competitive method. The advantage of this method is the ability to measure the KIE on the overall reaction (V_{max}) or on all steps up to and including the first irreversible one (V/K). The non-competitive method is generally limited to primary deuterium effects for two reasons. First, the magnitude of the effect is dependant upon the extent of substitution of the heavy

isotope, thus it is not reasonable to measure effects on trace labels. Secondly, the error is much larger for this method (2-5%) which limits its use with heavy atom or secondary effects (68). This shortcoming can be overcome with extreme experimental care and replicate measurements. Heavy atom effects on ^{15}N and ^{18}O have been directly measured with errors of less than 1% (77). These effects are normally measured using a continuous assay because time point assays can have errors of greater than 10% associated with them (68).

CHAPTER 2
SYNTHESIS AND CHARACTERIZATION OF SUBSTRATES

Introduction

The activated sugar nucleotide cytidine-5'-monophosphate-N-acetyl neuraminic acid (CMP-NeuAc, Figure 2-1) is the common donor substrate for the biosynthesis of sialylated glycoconjugates (78). The neuraminic acid portion of this donor molecule is transferred via sialyltransferases to a saccharide acceptor hydroxyl group on polysialic acids, glycoproteins, and gangliosides (79). An interesting feature of sialyltransferases is that while they differ in acceptor substrate specificity, all use CMP-NeuAc as the sialyl donor substrate (1). Thus, while this work focuses on the $\alpha(2\rightarrow6)$ and $\alpha(2\rightarrow3)$ sialyltransferases, much of it can be extended to the remaining members of the sialyltransferase family.

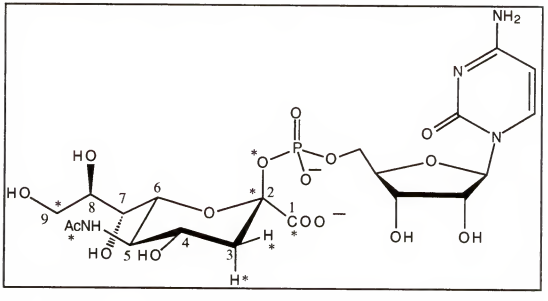


Figure 2-1. CMP-NeuAc: Asterisks denote sites of isotopic labeling.

Results

With this work focused on the characterization of the chemical behavior of the sialyltransferase donor substrate (CMP-NeuAc) as well as the enzyme catalyzed transfer reaction, it was necessary to synthesize a number of CMP-NeuAc isotopomers to carry out the desired experiments. This group of compounds consists of those possessing a radioactive trace label, those possessing a non-radioactive isotopic substitution, and some that have both a radioactive trace label as well and a non-radioactive

isotope label. The sites of isotopic substitution are shown in Figure 2-1. Compounds with radiolabels were prepared using the appropriate commercially available radiolabeled starting material. The isolated yields are listed below in Table 2-1.

CMP-NeuAc Isotopomer	Isolated %Yield
[9-^{3}H]	78
[9-^{3}H; 3,3'-^{2}H]	35
[1-^{3}H-N-acetyl]	40
[1-^{3}H-N-acetyl; 3,3'-^{2}H]	35
[1-^{14}C]	85
[1-^{13}C]	20
[2-^{14}C]	76
[1-^{14}C-N-acetyl]	86
[2-^{18}O; 1-^{3}H-N-acetyl] 80 atom%	31
[2-^{18}0] 53 atom%	11

Table 2-1. CMP-NeuAc isotopomers.

The investigation of the sialyltransferase mechanism necessitated the synthesis of an unnatural "slow" donor substrate that the enzyme could use instead of CMP-NeuAc. The required characteristics of a slow substrate are that it possesses a reduced value of V_{max}. However, the decrease in V_{max} should arise from a decrease in the rate of the chemistry step, not from a slow product off-rate. Our results indicate there is not a slow product off-rate associated with this system. Also, it is desirable if the alterations made to the substrate are remote from the site of chemistry on the molecule in order to limit the possibility of altering the chemistry catalyzed by the enzyme. The goal is simply to slow down the chemistry step relative to other steps in the mechanism. One molecule that satisfied these requirements was uridine-5'-monophosphate-N-acetyl neuraminic acid (UMP-NeuAc). This molecule was also synthesized containing isotopic substitutions. The isotopic labels of the UMP-NeuAc isotopomers synthesized were at positions analogous to those employed for CMP-NeuAc and are shown in Figure 2-2.

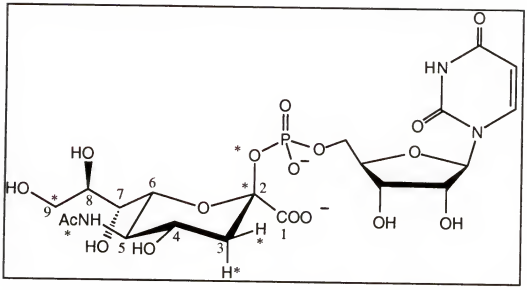


Figure 2-2. Uridine-5'-monophosphate N-acetyl neuraminic acid (UMP-NeuAc). Asterisks denote sites of isotopic labeling.

This compound was initially prepared in the same way CMP-NeuAc was with the exception that UTP was used in place of CTP. However, low yields (15%) and the requirement of a large amount of enzyme prompted the development of an alternative route in which CMP-NeuAc was synthesized and subsequently deaminated to give UMP-NeuAc. The isolated yields of the UMP-NeuAc isotopomers obtained from the deamination of CMP-NeuAc are

UMP-NeuAc radioisotopomer	% yield (CMP-NeuAc to UMP-NeuAc)
[9-^{3}H]	18
[2-^{14}C]	40
[^{14}C-NAc]	28
[^{3}H-NAc-3,3'-D_2]	38
[^{3}H-NAc]	33

Table 2-2. UMP-NeuAc radioisotopomers prepared by deamination of CMP-NeuAc.

presented in Table 2-2. Additionally, CMP-NeuAc and UMP-NeuAc were titrated with the results indicating that the carboxylate group has a $pK_a \leq 4$.

Two other slow substrate candidates were envisioned and the syntheses were attempted. These were cytidine-5'-monophosphate-3-deoxy-D-*manno*-nonulosonic acid and cytidine-5'-monophosphate-3,5-dideoxy-D-arabino-D-glycero-nonulosonic acid (Figure 2-3). The sugar portions of these molecules were successfully prepared, however, CMP-NeuAc synthase would not tolerate the modifications to form the link to CMP in either case.

This chapter will present the syntheses and the characterization of these substrates.

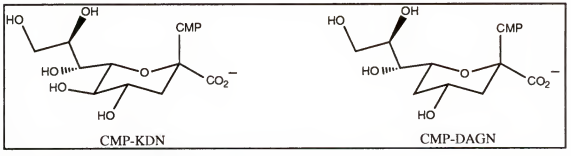


Figure 2-3. CMP-KDN and CMP-DAGN.

Discussion

Synthesis of CMP-NeuAc Isotopomers

Isotopomers of CMP-NeuAc were synthesized using the enzymatic and chemical methods shown in Figure 2-4. The positions of the isotopic labels are designated by asterisks. The first step of the synthesis employs the commercially available enzyme N-acetyl neuraminic acid (NANA) aldolase (80). This enzyme catalyzes an aldol condensation reaction between N-acetyl mannosamine (ManNAc) and pyruvate to give N-acetyl neuraminic acid (NeuAc) as the product. Isolated yields in the range of 75-85% were obtained. The equilibrium is forced toward the desired product by using an excess of the least expensive starting material. This turns out to be whichever starting material does not contain the radiolabel, or pyruvate if the desired product does not possess any radiolabels in the product. The desired radiolabeled NeuAcs are readily synthesized from the appropriate radiolabeled and commercially available starting materials. N-acetyl mannosamine is available with 6-^{3}H and ^{14}C-

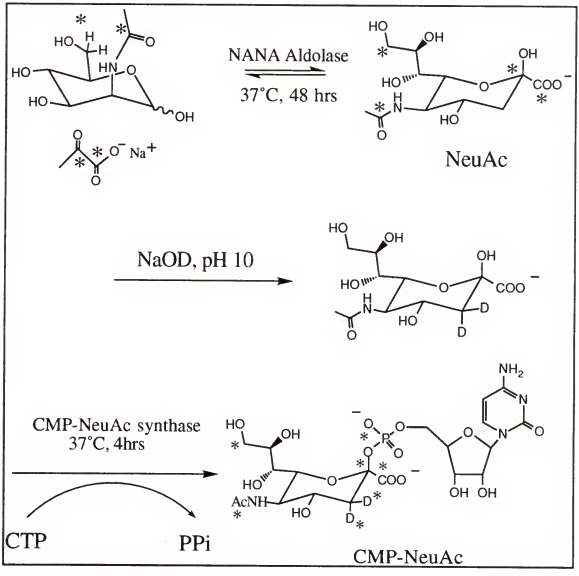


Figure 2-4. Synthesis of cytidine-5'-monophosphate N-acetyl neuraminic acid (CMP-NeuAc). Asterisks indicate sites possible isotopic substitution.

N-acetyl labels which give [9-^{3}H] and [^{14}C-N-acetyl] NeuAc, respectively. [^{3}H-N-acetyl] ManNAc was also prepared by Dr. Ben Horenstein which was subsequently used to synthesize [^{3}H-N-acetyl] NeuAc. NeuAc can be synthesized with labels at the anomeric carbon as well as the carboxylate carbon by beginning with [2-^{14}C] and [1-^{14}C] pyruvic acid to give [2-^{14}C] and [1-^{14}C] NeuAc, respectively. We have also prepared NeuAc with a ^{13}C label at the carboxylate carbon ([1-^{13}C] NeuAc) by beginning with [1-^{13}C] pyruvic acid as the starting material.

The progress of the NeuAc reaction can be monitored in two ways depending on the presence or absence of radiolabels. Those without radiolabels are monitored by ^{1}H-NMR (Figure 2-5) with the observation of a doublet of doublets at 1.8 ppm (C3, axial) and a doublet of doublets at 2.2 ppm (C3, equatorial) indicating the production of NeuAc (81). Integration of these peaks with respect to those of the starting material protons allows the reaction

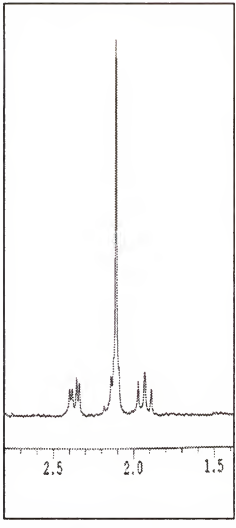


Figure 2-5. NeuAc ^{1}H-NMR: C3 and N-acetyl protons.

progress to be determined. Reactions containing radiolabels are monitored by analytical anion-exchange HPLC. The resulting

radiolabeled NeuAc products are carried through to subsequent steps, and nonlabeled NeuAcs are purified. Purification is accomplished by applying the reaction mixture to a column of Dowex 1X2-200 anion exchange resin (formate form) and eluted with a 0-2 M formic acid gradient. Fractions are collected and assayed for the presence of NeuAc by the thiobarbituric acid assay (TBA assay) (82).

NeuAc may be further isotopically modified once it has been isolated. Deuteriums may be introduced at the C3 position by exchange in alkaline deuterium oxide. This is possible due to the ability of the NeuAc ring to open as shown in Figure 2-6. The C3 protons are then acidic enough to be exchanged under alkaline conditions. This process occurs rapidly at a pH>12 with complete exchange in less than 1 hour at room temperature. Complete exchange is indicated by the loss of the two C3 proton resonances in the ^{1}H-NMR spectrum (83). It is also possible to specifically exchange either the axial or equatorial proton to yield the pro-R or pro-S NeuAc isotopomers, respectively (83).

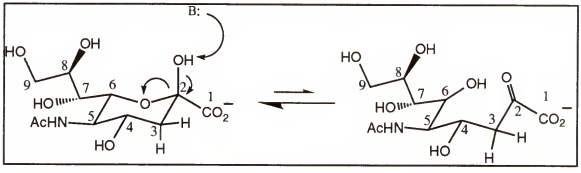


Figure 2-6. Ring opening mechanism.

By allowing the exchange to proceed under mildly alkaline conditions (pD 9), reasonable control over the rate of exchange can be gained. The axial proton is exchanged first. The reason for this specificity in the exchange is currently unclear. There may be interactions between functionality of the neuraminyl ring with the anomeric carbon when the ring opens that account for this. The progress of the exchange is monitored by the loss of the axial proton's resonance at 1.8 ppm and the collapse of the equatorial proton's resonance at 2.2 ppm to a doublet. This reaction was complete in 1 hour at room temperature at a pD of 9. The reaction is quenched by the addition of concentrated HCl to rapidly lower

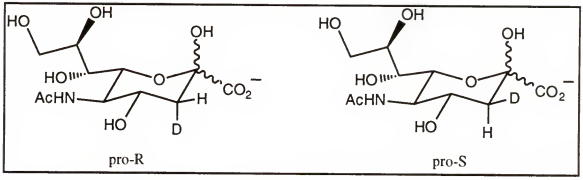


Figure 2-7. pro-R and pro-S NeuAc.

the pH. Once the pH is made neutral, the C3 protons are very stable and do not exchange appreciably. The % of each proton remaining can be determined by comparison of the integrals of the C3 protons relative to the N-acetyl protons of NeuAc. This procedure was accomplished with complete exchange of the C3 axial proton (pro-R), but was not completely selective. However, selective exchange has been reported (84). The equatorial proton was routinely observed to be approximately 15% exchanged. In order to prepare the pro-S compound, complete exchange of both C3 protons was first completed to give the dideuterio compound, followed by back exchange of H for D at the axial position in

alkaline H_2O. The reaction mixture is then quenched with concentrated HCl, rotovapped to dryness and brought back up in neutral D_2O in order to determine the % exchange by ^{1}H-NMR. However, the synthesis of pro-S CMP-NeuAc was not completed.

The oxygen of the anomeric hydroxyl of NeuAc was exchanged in the presence of $H_2{}^{18}O$ (pH > 12) to give [2-^{18}O] NeuAc. After ^{18}O exchange was estimated to be complete (2 hours, completion time estimated from experience with deuterium exchange), the mixture was neutralized with HCl and subsequently used in the synthesis of CMP-NeuAc. The presence of the ^{18}O label was verified by ^{1}H decoupled ^{31}P-NMR of the final CMP-NeuAc product (Figure 2-8). The single P peak of CMP-NeuAc is split into two peaks due to the isotopic splitting by $^{16}O/^{18}O$. The $\Delta\delta$ for these two peaks was 0.018 ppm. This compound was prepared with an equal amount of each oxygen isotope for positional isotope exchange (PIX) experiments as well as with the goal of complete ^{18}O incorporation for the leaving group ^{18}O KIE. The

material used for the leaving group KIE also contained a ^{3}H label in the N-acetyl group of NeuAc as a trace label. Unlabeled NeuAc was added to the reaction mixture prior to ^{18}O exchange to give enough material for ^{31}P-NMR analysis of the % incorporation of ^{18}O at the anomeric carbon.

With the desired NeuAc isotopomer in hand, the second step in the reaction scheme shown in Figure 2-4 is catalyzed by CMP-NeuAc synthase (85). The reaction is initiated by adding the enzyme and CTP. The reaction proceeds at 37 °C (pH 7.5) and becomes acidic as CMP-NeuAc is produced. It is kept neutral by manual addition of NaOH. Under these conditions, any labeling that has been previously performed on NeuAc will be stable and is "locked in" once CMP-NeuAc is formed. The production of CMP-NeuAc can be detected by the change in chemical shifts of the C3 protons in the ^{1}H-NMR spectrum. The axial proton appears as a triplet of doublets (6 peaks) shifted upfield to 1.6 ppm (from 1.8 ppm), while the equatorial proton remains a doublet of doublets

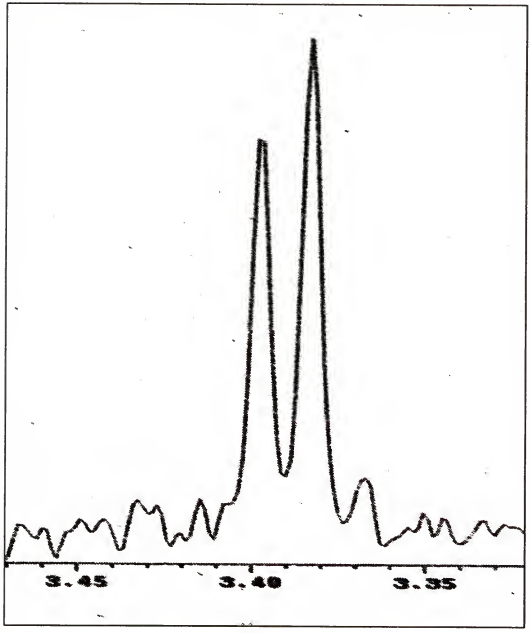


Figure 2-8. ^{31}P-NMR of [2-^{18}O] CMP-NeuAc.

shifted downfield to 2.4 ppm (from 2.2 ppm). Reaction progress may also be monitored by HPLC using a UV/VIS detector by integrating the peaks for reactant (CTP) and product (CMP-NeuAc) in the HPLC chromatogram. If the reaction involves radioactive reactants, progress is monitored by collecting fractions from the HPLC and assaying for radioactivity by liquid scintillation counting. When the reaction is judged to be complete by these methods, the product can be purified by anion exchange HPLC. Product containing eluent from the HPLC must then be desalted to remove NH_4HCO_3. This is accomplished by using Amberlite IR120-H^+ cation exchange resin as described in the Experimental section.

Synthesis of Uridine-5'-Monophosphate Neuraminic Acid (UMP-NeuAc)

The synthesis of UMP-NeuAc begins with NeuAc as the starting material. NeuAc may be isotopically manipulated in the same fashion described above. There are two routes to UMP-NeuAc from this point. The first is to use CMP-NeuAc synthase

with UTP in place of CTP (86). The differences in this procedure versus the synthesis of CMP-NeuAc are that CMP-NeuAc synthase is desalted before being used in the reaction and larger quantities of enzyme are used. The enzyme is stored in an ammonium sulfate solution as a stable precipitate, and it was noticed that desalting improved reaction yields. This phenomenon may result from the enzyme being less stringent in its substrate specificity under low salt conditions. Yields for this reaction still remained low, usually around 15% for the NeuAc to UMP-NeuAc step as opposed to above 80% regularly observed for CMP-NeuAc. Due to these low yields, an alternate route to UMP-NeuAc was explored. This alternate approach involved chemical deamination of CMP-NeuAc to give UMP-NeuAc.

The two reagents tested in this strategy were sodium bisulfite (87) and sodium nitrite. Precedence for the sodium nitrite route was seen in the deamination of cytosine to uracil by

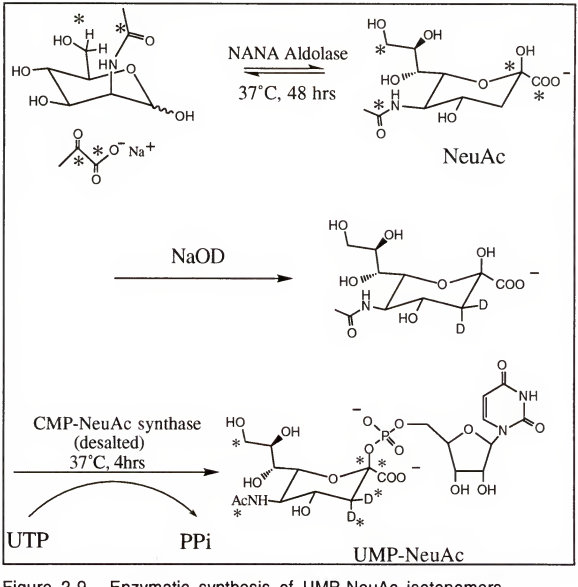


Figure 2-9. Enzymatic synthesis of UMP-NeuAc isotopomers.

nitrous acid (88). Reactions were run by adding the appropriate reagent to a solution of CMP-NeuAc and acidifying with 1N HCl to a pH of 4.5.

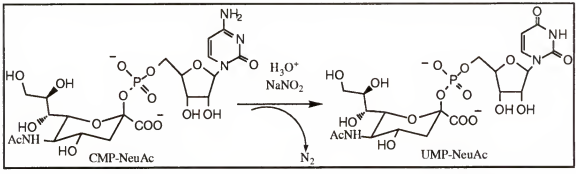


Figure 2-10. Chemical deamination of CMP-NeuAc to UMP-NeuAc by sodium nitrite.

Reactions were initially run at room temperature or at 37 °C. Difficulty in this approach arose due to the inherent lability of the sugar-phosphate bond of CMP-NeuAc under acidic conditions resulting in significant decomposition of CMP-NeuAc (86, 89). The production of UMP-NeuAc, as well as the decomposition of CMP-NeuAc was followed by analytical HPLC on a MonoQ anion exchange column. From the initial test reactions, it was demonstrated that sodium nitrite had potential to be a suitable reagent for this work, while bisulfite did not give the desired product. Chromatograms of the bisulfite reactions

showed only decomposition products, while a new peak appeared in the sodium nitrite reactions which co-eluted with UMP-NeuAc synthesized enzymatically. Another physical observation indicating the occurrence of deamination was the presence of bubbles in the reaction mixture seen immediately upon acidification, presumably from the production of N_2 gas. The reaction conditions were not favorable because a significant amount of decomposition of CMP-NeuAc had taken place. This problem was circumvented by allowing the reactions to proceed at 4 °C. As the reaction proceeds it becomes basic and the pH is maintained by the manual addition of 1N HCl. Under these conditions a reaction consisting of 1mM CMP-NeuAc and 1M $NaNO_2$ resulted in a mixture consisting of 44% CMP-NeuAc, 39% UMP-NeuAc, 9% CMP, and 8% UMP after 48 hours. These yields were calculated from integration of the peaks corresponding to these compounds in the HPLC chromatogram (values were corrected for the respective epsilon values of CMP and UMP). This result was extremely promising because it offered the possibility to

synthesize UMP-NeuAc in higher yield without significant loss of CMP-NeuAc starting material to acid catalyzed decomposition. Additionally, any unreacted CMP-NeuAc could be recovered during purification of the desired product (UMP-NeuAc) with unreacted nitrite being removed. This material could then either be used in experiments requiring radiolabeled CMP-NeuAc, or recycled and used again in deamination reactions. This reaction was then carried out on radiolabeled materials in which the reaction procedure was monitored by HPLC and liquid scintillation counting (results in Table 2-2).

Titration of CMP-NeuAc and UMP-NeuAc

One of the mechanistically interesting properties of the donor substrate (CMP-NeuAc) is the role the carboxylate may play in the chemical reactivity of CMP-NeuAc. The carboxylate may function as a general base to facilitate nucleophilic attack via deprotonation of an incoming nucleophile as shown in Figure 2-11 (90). Another role the carboxylate could play is that of an

electrostatic catalyst by stabilizing a transition-state or intermediate with oxocarbenium ion character (86, 91). A third

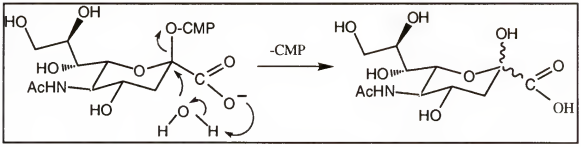


Figure 2-11. Carboxylate facilitating nucleophilic attack by acting as a general base.

possibility is that the carboxylate itself may act as a nucleophile, and form an intermediate alpha lactone. Although this intermediate would be considerably strained, its occurrence is a distinct possibility. Carboxylate nucleophilic assistance might aid in the displacement of the leaving group. Evidence for this was presented by Sinnott concerning aryl sialoside hydrolysis. The magnitude of deuterium isotope effects were seen to be pH sensitive. This was attributed to the protonation

state of the carboxylic acid, with smaller isotope effects seen at a higher pH due to the presumed ability of the carboxylic acid to participate nucleophilically because it was deprotonated. Conversely, larger isotope effects were seen at a lower pH where the carboxylate was protonated and unable to participate. When the carboxylic acid was able to participate as a nucleophile, it would stabilize the positive charge build-up at the reaction center, thus lowering the deuterium isotope effects (92). A hypothetical mechanism for this occurrence in the hydrolysis of CMP-NeuAc is illustrated in Figure 2-12. It is considered unlikely that the sialyltransferase mechanism involves an alpha lactone due to the inversion of stereochemistry at the anomeric carbon. Additionaly, our methanol trapping experiments discussed in Chapter 5 argue against alpha lactone formation in the solvolysis of CMP-NeuAc. Equal trapping on both faces of the anomeric carbon was observed. A fourth possibility is that the carboxylate is functioning as a general acid by donating a proton to the leaving phosphate group. This is illustrated in Figure 2-13.

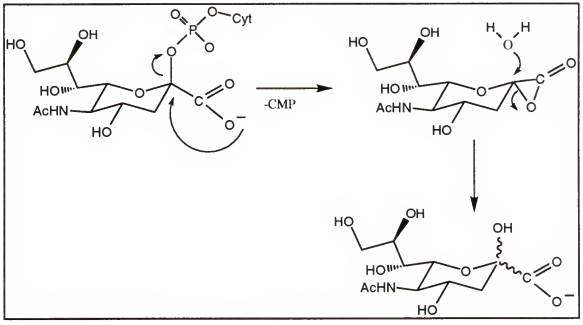


Figure 2-12. Carboxylate facilitating nucleophilic attack by forming the alpha lactone.

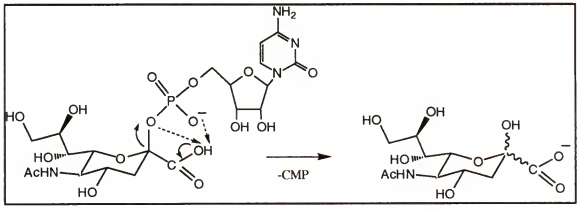


Figure 2-13. Carboxylate functioning as a general acid. It is unclear which oxygen would be protonated.

While a number of our experiments address these possibilities, an essential piece of information needed to interpret the data is the pK_a of the carboxylate. The protonation state of the carboxylate under the experimental conditions must be known to help determine the possible roles it could play.

A solution of CMP-NeuAc was titrated with HCl in the pH range of 3-8 resulting in the curve presented in Figure 2-14. A difficulty in this experiment arose due to the fact that CMP-NeuAc is very acid labile and begins to decompose at low pH (89). Thus, the measurements were made at 4 °C to avoid the titration of NeuAc and CMP (decomposition products) along with CMP-NeuAc. The resulting titration curve showed a titratable group with an apparent pK_a of 4.8. While this was most likely the amino functionality of cytidine, it was unclear from the titration curve if the observed pK_a was due to a single group. A possibility was that the carboxylate was also being titrated in this region.

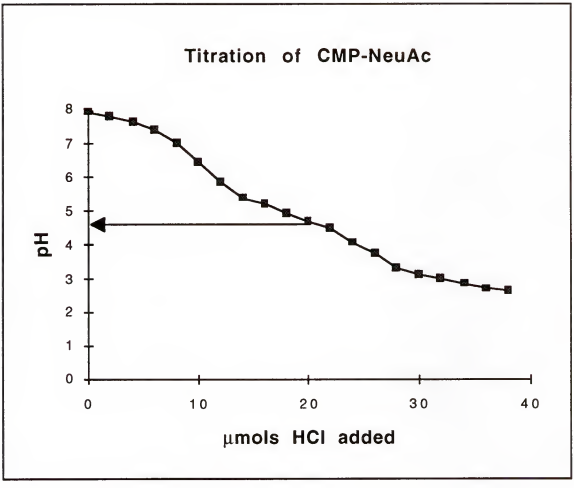


Figure 2-14. Titration of CMP-NeuAc. The solid line connects individual data points and indicates the change in pH with respect to the addition of acid.

Two approaches were employed to titrate only the carboxylate group. The first was to repeat the titration using

UMP-NeuAc. This compound is identical to CMP-NeuAc with the exception of the carbonyl group in place of the amino group in the nucleotide ring. Therefore, the titration curve should show no titratable groups if the only group observed in the titration of CMP-NeuAc was the amino group of cytidine. The carboxylate group pK_a should not be perturbed by the change in the nucleotide ring due to its distance from the reaction center. An initial attempt at titrating UMP-NeuAc was hindered by the presence of carbonic acid. Carbonic acid was produced when HCl was added to the mixture because of remnant NH_4HCO_3 resulting from incomplete desalting. This problem was circumvented by repurifying UMP-NeuAc by HPLC using a Tris buffer system. The titration curve for UMP-NeuAc is shown in Figure 2-15. This curve showed no titratable groups. From this curve we can see that a single titratable group with an apparent pK_a of 4.8 has been removed.

The second approach to titrating the carboxylate was to synthesize [1-^{13}C] CMP-NeuAc and perform the titration by following the change in chemical shift of the carboxylate carbon

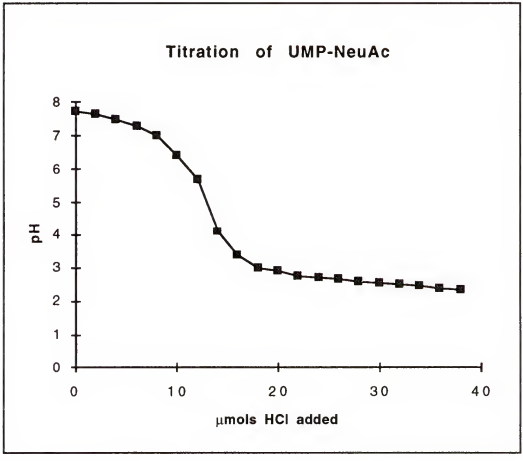


Figure 2-15. Titration of UMP-NeuAc. The solid line connects the individual data points and indicates the change in pH with respect to the addition of acid.

in the ^{13}C- NMR spectrum. It is documented in the literature that the chemical shift of a carboxylate will be shifted upfield when it is protonated (93). The ^{13}C enrichment of the carboxylate carbon allowed the NMR spectrum to be aquired in a relatively short time (3 minutes), and only a single peak was observed. This experimental approach also alleviated the problem of decomposition under acidic conditions due to the observation that the carboxylate carbons of CMP-NeuAc and NeuAc have different chemical shifts. Thus, it was possible to follow the carboxylate carbon of CMP-NeuAc during the titration without worrying about the decomposition products affecting the result. An end point was not reached during this experiment due to the rapid acid catalyzed hydrolysis of UMP-NeuAc, but the curve does show a single titratable group with an estimated pKa $\leq$ 3.4. The carboxylate carbon chemical shift was seen to be 174.4 ppm in

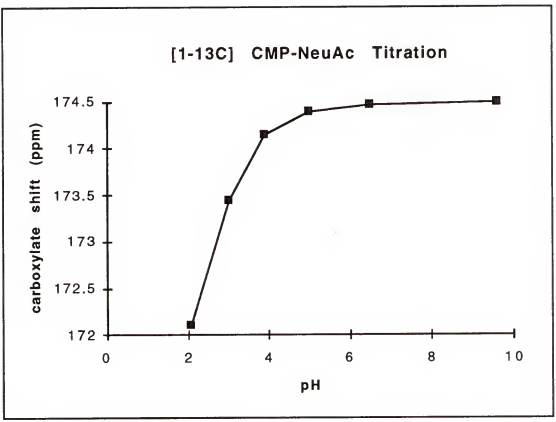


Figure 2-16. ^{13}C-NMR titration of [1-^{13}C] CMP-NeuAc.

the pH range of 5-10, and was shifted upfield upon protonation to a final value of 172.1 at pH 2. These experiments gave us confidence that the pK_a of the carboxylate group was less than or equal to 4. The majority of the kinetic experiments were carried out at pH 5 or greater, thus reflecting the behavior of an ionized

carboxylate group. While the possibility that the reactions proceed via a minor portion of CMP-NeuAc in which the carboxylate is protonated, this seems unlikely.

Synthesis of CMP-KDN and CMP-3,5-dideoxy-D-arabino-D-glycero-nonulosonic acid

As previously mentioned, attempts were made to synthesize other slow substrates for sialyltransferase. The two molecules in Figure 2-3 were considered to be possible candidates due to their difference in functionality on the sugar ring compared to NeuAc, but identical in nature around the reaction center. It was anticipated that this might result in the unfavorable positioning of the substrate in the active-site pocket, thus altering catalytic efficiency and thereby decreasing V_{max}. The first step in the synthesis was carried out the same way the NeuAc synthesis was. The reaction was catalyzed by NANA aldolase which tolerates variance on the sugar ring (94). The yields for these reactions were lower than those observed for NeuAc. KDN was produced

with a 60% yield, while DAGN was produced with a 65% yield as indicated by ^{1}H-NMR analysis in a manner analogous to that described for NeuAc. These products were taken through to the second step without purification. The literature indicated that CMP-NeuAc synthase was strict in its substrate specificity and would not tolerate modification in the NeuAc ring (95), however, the possibility remained that with sufficient enzyme, useful amounts of CMP-KDN and CMP-DAGN might be obtained. These reactions were followed by HPLC. The appearance of a new product peak was seen for both reactions, but in a very low yield (2-3% by HPLC analytical injection). The compounds were purified by HPLC and tested as substrates for $\alpha(2\rightarrow6)$ sialyltransferase, but no activity was observed. No further kinetic characterizations were performed.

Experimental

Materials

Buffers and reagents were purchased from Sigma and Fisher. NANA aldolase was purchased from Shinko American. Rat liver $\alpha(2\rightarrow6)$ sialyltransferase was purchased from Sigma and Boehringer. Recombinant rat liver $\alpha(2\rightarrow3)$ sialyltransferase was purchased from Calbiochem. N-acetyl mannosamine isotopomers ([6-^{3}H] and [1-^{14}C N-acetyl]) and sodium pyruvate ([1-^{14}C]) were purchased from New England Nuclear and Moravek. D_2O was obtained from Aldrich or Cambridge Isotope Laboratories, Inc. and was 99.9% deuterium. Cytidine and Uridine triphosphate (CTP and UTP) were purchased from Sigma as the disodium salt with 2.5 equivalents of hydration. Liquid scintillation fluid (ScintiSafe 30%) was purchased from Fisher. Plasmid pWV200B harboring the expression construct for *E. coli* CMP-NeuAc synthase was a gift from Dr. W. F. Vann at the National Institutes of Health. Protein concentrations were determined by the BioRad version of the Bradford dye-binding assay (96).

Instrumental

HPLC was performed on a Rainin HPXL gradient unit interfaced to a Macintosh personal computer. Separations were performed on a MonoQ HR10/10 anion exchange column (Pharmacia). A Rainin Dynamax UV-1 detector was employed to monitor separations at 260 nm. Liquid scintillation counting was performed using a Packard 1600 TR instrument which dumped data to a floppy disk for subsequent analysis on a personal computer. An Orion Sure-Flow pH probe was used in conjunction with an Accumet model 15 pH meter for all pH measurements. A Rainin-Dynamax fraction collector (Model FC-1) was used to collect eluent samples from the HPLC. NMR spectra were recorded on a Gemini 300 MHz NMR and data was subsequently worked up on a Unix Sun station. Cells were lysed using a French pressure cell with a Carver hydraulic press. Centrifugation was performed with a Sorvall RC 5B centrifuge.

Synthesis of NeuAc

Sodium pyruvate(3.76 g, 34 mmol), N-acetyl mannosamine (752 mg, 3.40 mmol) and 15 U NANA aldolase were incubated at 37 °C in 45 mL 40 mM phosphate buffer (pH 7.5) which contained 30 mg sodium azide and 90 mg BSA for 48 hours. NeuAc was purified from the reaction mixture by anion exchange chromatography (Dowex 1X2-200, formate form) employing a 0-2N formic acid gradient. NeuAc containing fractions were detected by the thiobarbituric acid assay (82) and pooled. Pooled fractions were rotovapped to dryness giving 848 mg (2.75 mmols) of NeuAc as a white solid (81% yield). At this point dideuterio NeuAc could be prepared by dissolving vacuum dried NeuAc into alkaline D_2O (pD > 12). Both C3 protons are exchanged under these conditions at room temperature in less than 1 hour.

Overexpression of CMP-NeuAc Synthase

The overexpression of CMP-NeuAc synthase was made possible through the gift of an *E. coli* clone containing a CMP-

NeuAc synthase expression plasmid from Dr. W. F. Vann (95). A single colony from a freshly streaked plate of *E. coli* JM105 harboring pWV200B was picked from the plate with a sterile toothpick and placed in 5 mL Luria Broth containing 100 μg/mL ampicillin. This was placed in the 37 °C shaker 9.5 hours until OD_{600}= 1 was reached. A 1 mL aliquot was then removed and used to inoculate 50 mL Luria Broth containing 100 μg/mL ampicillin. The 50 mL culture was placed in the 37° C shaker for 4.5 hours until OD_{600}= 1.1. Two 1 liter cultures of luria broth containing 100 μg/mL ampicillin were then each inoculated using 10 mL of the 50 mL culture and placed in the 37 °C shaker for 22 hours until OD_{600}= 1.4. The cultures remained in the 37 °C shaker an additional 16 hours after the addition of 165 mg of IPTG (0.692 mmols, 13.8 mM) added to each to induce overexpression. The cells were harvested at 5000 RPM, 6 °C, 30 minutes to harvest the cells and the protein was purified following the published protocol which is briefly outlined below (97). The supernatant was decanted and the cell pellets were resuspended in 10 mL

purification buffer (50 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, pH 7), and were lysed using the French pressure cell (2 runs) and Carver hydraulic press. The lysed cells were spun at 8000 RPM, 6 °C, 25 minutes. The supernatant was applied to an Orange-A affinity column (1.5 X 15 cm) pre-equilibrated in purification buffer (described above) at 4 °C. The column was washed with low salt buffer (300 mL) until no more protein was eluted as detected by the Bradford protein assay (96). A 0-1 M KCl gradient was then begun (300 mL total volume). Fractions were collected (10 mL) and assayed for protein by the Bradford assay. Protein containing fractions were combined and concentrated using an Amicon filtration concentrator until a protein concentration >10 mg/ml was reached. The enzyme solution was then made 80% $(NH_4)_2SO_4$. The enzyme was stored as the $(NH_4)_2SO_4$ precipitate at 4 °C until further use. The enzyme was estimated to be >95% pure by SDS-PAGE analysis. The enzyme was routinely purified with a specific activity of 2 U/mg. The specific activity was determined by a coupled assay in which

pyrophosphate produced from the coupling of CTP and NeuAc was taken to phosphate with alkaline pyrophosphatase. The amount of phosphate produced was quantified by the Malachite Green assay (98).

Synthesis of CMP-NeuAc (99)

NeuAc (25 mg, 0.081 mmol), CTP (56 mg, 0.12 mmol), and CMP-NeuAc synthase (1 Unit) were incubated at 37 °C for 3 hours in 50 mM Tris-HCl, 50 mM $MnCl_2$, pH 7.5 buffer. During the course of the reaction, a precipitate was formed (presumably the magnesium salt of pyrophosphate) and the reaction became acidic. The pH was kept neutral by manual addition of 1N NaOH. Prior to purification the reaction was centrifuged in a 1.5 mL polypropylene centrifuge tube (8,000 RPM; 10 minutes). The product was purified by HPLC (Mono Q anion exchange column) giving 0.061 mmols (75% yield). Radiolabeled CMP-NeuAc isotopomers were synthesized in the same manner with enzyme

and reagents scaled accordingly using the appropriately radiolabeled precursors to yield the desired product.

HPLC Purification and Desalting of Products

CMP-NeuAc and UMP-NeuAc were purified by HPLC on a Mono Q HR 10/10 anion exchange column using 100mM NH_4HCO_3, 15% methanol, pH 8.0. An exception to this was reactions with radiolabeled pyruvate. These reactions were purified with 75 mM NH_4HCO_3 to separate pyruvate from CMP-NeuAc. Product containing fractions were detected by UV/VIS and collected. The eluent was subsequently desalted using Amberlite IR120-H^+ cation exchange resin. The Amberlite resin was prepared in 100 g batches by first washing with 500 mL 95% ethanol followed by 400 mL 4N HCl. The resin was then washed with a copious amount of water (usually 1-2 L) until the filtrate was neutral by pH paper. The resin was then stored at 4 °C and always washed with water again immediately before use. Amberlite was then added directly to product containing fractions which were combined in a

sealed conical tube which had been on ice. These were then vortexed three times ≈ 20 s each time. The cap was removed from the conical tube after each vortexing to release CO_2 produced during desalting. A caveat of this process is that solutions often became acidic. Best results were seen when ≈ 2 g of Amberlite were used with a volume of 15-20 mL. Solutions with lower $[NH_4HCO_3]$ also seemed to become acidic less often. Immediately after vortexing was complete, the mixture was filtered through glass pipettes containing glass wool directly into an appropriately sized round bottom flask. The remaining resin was then washed twice with ice cold water, and the washings were filtered through the pipettes into the round bottom flask. Desalted solutions were then concentrated *in vacuo* without heating and redissolved in water. Aliquots of the purified material could then be checked for purity by HPLC. Completion of desalting was established by assay for ammonium ion (100). Purified CMP-NeuAc was stored in aqueous solution at -20 °C.

Synthesis of pro-S NeuAc

NeuAc (50 mg, 0.162 mmol) was brought up in 1.5 ml D_2O, and the pD was adjusted to 11.5 with 2N NaOD and placed in an NMR tube for analysis. Complete exchange of both C3 protons was seen after 20 hours. The completely exchanged product was concentrated *in vacuo* at room temperature and brought up in H_2O. The pH was adjusted to 11.5 with 2N NaOH and the reaction was allowed to proceed at room temperature. The reaction was neutralized to pH 7 with 1N HCl after 6 hours. ^{1}H-NMR analysis indicated 85% back exchange of the C3 axial proton, with no back exchange of the equatorial proton by integration of the C3 proton resonances.

Synthesis of [^{3}H-N-acetyl]pro-R CMP-NeuAc

[^{3}H-N-acetyl] NeuAc (0.016 mmol; 0.924 Ci/mol) was dissolved in 750 μL of 50 mM sodium phophate/D_2O buffer (pD 12.0), placed directly in a 9 inch NMR tube and immediately frozen

in dry ice/acetone. The tube was then flame sealed under vacuum and swiped to check for radioactive contamination. The contents of the tube were thawed prior to taking the first ^{1}H-NMR spectrum. The exchange of the axial proton at C-3 by deuterium was followed by ^{1}H-NMR. The ^{1}H-NMR spectrum indicated that the axial proton was >95% exchanged after 80 minutes at 25°C, while the equatorial proton was 10-15% exchanged. The contents of the tube were immediately removed and place in a 1.5 mL microfuge tube along with 75 µL of 1N HCl to quench the exchange. The mixture was subsequently rotovapped to dryness *in vacuo*. The dried material was brought up in 400 µL 100 mM $MnCl_2$, 50 mM Tris, pH 7, containing CTP (0.021mmol). The reaction was initiated by the addition of 0.3 units of CMP-NeuAc synthase and was placed at 37°C for 2.5 hours when it was judged to be complete by analytical HPLC (MonoQ anion exchange) and liquid scintillation counting. The product CMP-NeuAc was then purified by HPLC and desalted with Amberlite IR-120H$^+$ cation

exchange resin. The purified product was then rotovapped to dryness *in vacuo* and brought up in 750 μL D_2O. This was placed in a 9 inch NMR tube, frozen in dry ice/acetone, and flame sealed under vacuum. The ^{1}H-NMR spectrum indicated that the axial proton was >95% exchanged, while the equatorial proton was 10-15% exchanged for deuterium, thus no isotopic exchange occured in the second step. The desired [^{3}H-N-acetyl] CMP-NeuAc was obtained in 23% yield (3.5 μCi).

Synthesis of 50 atom % [2-^{18}O] CMP-NeuAc

N-Acetyl neuraminic acid (35 mg, 0.114 mmol) was dissolved in 200 μL of 1.9 mM glycine, 0.5 M $MnCl_2$ buffer (pH>12) and 300 μL of 97.5% $H_2{}^{18}O$ was then added. The reaction mixture was placed at 37 °C for 12 hrs. After the exchange, cytidine-5'-triphosphate (75 mg, 0.165 mmol) was directly added to the reaction mixture. The reaction mixture became acidic and was readjusted to pH 8 with 25 μL 10 N NaOH. CMP-NeuAc synthetase

(0.5 units) was then added and the reaction mixture was placed at 37 °C for 3 hrs. The reaction progress was monitored by analytical HPLC injections on a MonoQ anion exchange column based on the integration areas of the CMP-NeuAc and CTP peaks. After completion, the reaction mixture was purified by HPLC on the MonoQ column. Product containing fractions were desalted by vortexing with Amberlite IR-120 H^+ cation exchange resin as described earlier. The solution was then rotovapped to dryness *in vacuo* and brought up in 800 μL D_2O. The solution was made 10 mM in EDTA to chelate species which might cause the phosphorous peak to broaden. The ^{1}H-decoupled ^{31}P-NMR spectrum was indicated the presence of ^{18}O as evidenced by two peaks for the ^{18}O and ^{16}O species with a $\Delta\delta$ of 0.018 ppm. The relative areas of the ^{18}O and ^{16}O peaks were 0.53 and 0.47, respectively, indicating that the product contained 53% ^{18}O incorporation. The product was obtained in a 49% overall isolated yield.

Synthesis of [^{3}H-N-acetyl; ^{18}O] CMP-NeuAc

[1-^{14}C N-acetyl] ManNAc (30μCi, 10 mCi/mmol) was dissolved in 200 μL of 40 mM phosphate, 2 mg/ml BSA, 2 mg/ml NaN_3 buffer (pH 7.5) along with 5 mg pyruvic acid (0.056 mmols) and 1 unit neuraminic acid aldolase. This mixture was placed at room temperature for 4 days and judged to be >85% complete by LSC counting of HPLC fractions (50 mM NH_4HCO_3, 15% methanol, 2 mL/min, 2 mL fractions collected) containing [^{14}C] ManNAc (r.t. 3 min) and [^{14}C] NeuAc (r.t. 6 min). The mixture was then rotovapped to dryness *in vacuo* and brought up in 250 μL of 1.9 mM glycine, 0.5 M $MgCl_2$, pH 9.4 buffer. An additional 10 mg unlabeled NeuAc was added to allow the final ^{18}O incorporation to be determined later by ^{31}P-NMR. This mixture was rotovapped to dryness *in vacuo* and brought back up in 200μl $H_2{}^{18}O$. The pH was found to still be ≈9.4 by pH paper. This solution was placed at 37 °C for 12 hours to exchange in ^{18}O. CTP (10 mg, 0.021 mmol) was

added along with 0.5 units of CMP-NeuAc synthase before placing the reaction at 37 °C for 2 hours. The reaction progress was monitored by HPLC. After being judged to be complete, the product was purified by HPLC on a MonoQ column. The CMP-NeuAc fraction was collected, desalted with Amberlite IR-120H$^+$, and rotovapped to dryness *in vacuo*. The material was then brought up in 800 μL D_2O and then made 10 mM in EDTA. The product fraction contained 4 μCi of product for a 30% yield. Integration of the ^{16}O and ^{18}O phosphorous peaks indicated that there was 83% ^{18}O incorporation in the final product ($\Delta\delta$=0.018).

Enzymatic Synthesis of UMP-NeuAc

NeuAc (10 mg, 0.032 mmol), UTP (20 mg, 0.041 mmol), and desalted CMP-NeuAc synthase (1.5 units) were incubated at 37 °C for 8 hours in 250 μL 50 mM Tris-HCl, 50 mM $MnCl_2$, pH 7.5 buffer. The yield by HPLC analysis prior to purification was 18.5%. The UV-VIS spectrum showed lambda max=252 nm. The

product was acid labile like CMP-NeuAc, but hydrolyzed to form UMP. ^{1}H-NMR (D_2O) 7.79(d,J=8.1, 1H, H_6-uracil), 5.82(d,J=4.5,1H, H_1-ribose), 5.78(d,J=8.1, 1H, H_5-uracil), 4.22-4.18(m), 4.12-4.04 (m) 3.98(d,J=10.8), 3.91(m, 1H), 3.82-3.7(m), 3.52-3.42(m), 3.31(d,J=9.9, 1H), 2.32(dd,J=4.5,13.2, 1H, H_{3eq}, NeuAc), 1.90(s, 3H, NCOCH_3), 1.49(m, 1H, H_{3ax}, NeuAc).

Desalting of CMP-NeuAc Synthase

The protein solution (1 mL, 4 mg, 1 unit) in 80% ammonium sulfate was centrifuged at 12,000 rpm for 2 minutes. The supernatant was decanted and the protein pellet was brought up in 2 mL of 200 mM Tris-HCl, 20 mM $MnCl_2$, 200 mM DTT, 10% glycerol, pH 7.0 buffer. This solution was placed in a Centriprep concentrator with a molecular weight cutoff of 10,000 Da (CMP-NeuAc synthase is 45,000 Da). This was spun at 5000 rpm, 20 min, 4 °C. The ultrafiltrate that goes through the membrane was decanted and 2 mL buffer was added to the concentrated solution. This was done four times to desalt the solution. The protein was

brought to a final volume of 1.5 mL in the buffer before being used in reactions.

Chemical Synthesis of UMP-NeuAc

CMP-NeuAc (25 mg, 0.041 mmols) was dissolved in 250 μL dH_2O. This solution was made 1 N with $NaNO_2$ and adjusted to pH 4.5 with 1 N HCl. The reaction mixture was placed at 4 °C for 48 hours. The pH was monitored by pH paper and maintained at 4.5 by manual addition of 1 N HCl. The reaction proceeds to 40% completion with less than 10% hydrolysis of starting material in this time. The ^{1}H-NMR spectrum is identical to that of enzymatically synthesized UMP-NeuAc. The appropriate isotope-substituted CMP-NeuAc was used to synthesize the desired UMP-NeuAc isotopomers in the manner described above.

Synthesis of CMP-KDN

D(+)-mannose (180.2 mg; 1 mmol) was combined with pyruvic acid (1.1 g; 10 mmol) in 10 ml 50 mM Na phosphate, 1mM

DTT, 2 mg/mL BSA, 2 mg/mL NaN_3, pH 7.4 buffer. The reaction was initiated by the addition of 10 units NeuAc aldolase and placed at 37 °C for 5 days. Formation of KDN was indicated by the appearance of ^{1}H-NMR resonances at 1.8 ppm (triplet) and 2.2 ppm (quartet) diagnostic for sialic acids. These resonances were absent from the starting material ^{1}H-NMR spectrum. The reaction was judged to be 65% complete by integration of ^{1}H-NMR peaks. A 500 μL aliquot (8.7 mg, 0.0325 mmol @ 65% completion) was directly withdrawn from this reaction and used in the next step. The solution was made 50 mM in Tris and 100 mM in $MnCl_2$ at pH 7.5 and then 30 mg of CTP (0.062 mmol) was added. The reaction was initiated by the addition of 1.5 units CMP-NeuAc synthase and placed at 37 °C for 17 hours. The reaction was monitored by analytical HPLC injections with a UV/VIS detector. A new peak was detected and purified. However, the compound was not a substrate for α(2→6) sialyltransferase and was not characterized beyond its UV/VIS spectrum. Its utility as a sialyltransferase

substrate was tested by preparing 30 μL of a 400 μM solution of the purified product along with 16 mM LacNAc in 40 mM cacodylate, 0.2 mg/mL BSA, NaN_3, pH 7 buffer. The reaction was initiated by the addition of 2 mU α(2→6) sialyltransferase and run at 37 °C for 30 minutes. Product formation was analyzed by analytical HPLC (MonoQ anion exchange). No turnover by the enzyme was observed.

Synthesis of CMP-3,5-dideoxy-D-arabino-D-glycero-nonulosonic acid (CMP-DAGN)

2-Deoxy glucose (164.2 mg; 1 mmol) was combined with pyruvic acid (1.1 g; 10 mmol) in 10 mL 50 mM Na phosphate, 1 mM DTT, 2 mg/mL BSA, 2 mg/mL NaN_3, pH 7.4 buffer. The reaction was initiated by the addition of 10 units NeuAc aldolase and placed at 37°C for 5 days. Formation of DAGN was indicated by the appearance of ^{1}H-NMR resonances at 1.8 ppm (triplet) and 2.2 ppm (quartet) diagnostic for the C3 protons of sialic acids. These

resonances were absent from the starting material ^{1}H-NMR spectrum. The reaction was judged to be 50% complete by integration of ^{1}H-NMR peaks. A 500 μL aliquot (6.3 mg, 0.025 mmol @ 50% completion) was directly withdrawn from this reaction and used in the next step. The reaction was made 50 mM in Tris and 100 mM in $MnCl_2$ at pH 7.5 and then 30 mg CTP (0.062mmol) was added. The reaction was initiated by the addition of 1.5 units CMP-NeuAc synthase and placed at 37 °C for 17 hours. The reaction was monitored by analytical HPLC injections with a UV/VIS detector. A new peak was detected and purified. However, the compound was not a substrate for α(2→6) sialyltransferase and was not characterized beyond UV/VIS spectrum. Its utility as a sialyltransferase substrate was tested by preparing 30 μL of a 400 μM solution of the purified product along with 16 mM LacNAc in 40 mM cacodylate, 0.2 mg/mL BSA, NaN_3, pH 7 buffer. The reaction was initiated by the addition of 2 mU α(2→6) sialyltransferase and run at 37 °C for 30 minutes.

Product formation was analyzed by analytical HPLC (MonoQ anion exchange). No turnover by the enzyme was observed.

^{13}C-NMR Titration of 1-^{13}C-CMP-NeuAc

[1-^{13}C] CMP-NeuAc in 750 µL D_2O (250 mM) was brought to pH 9.4 with 1 N NaOH and placed in the NMR tube. The carbon NMR was then aquired (1024 scans). The solution was withdrawn and placed in a 1.5 mL microfuge tube on ice while the pH was adjusted to 6.5 with 1 N HCl. The mixture was placed back in the NMR tube and the spectrum was aquired again (512 scans). This process was continued by aquiring spectra at pH 4.8, 3.9, 3.0, and 2.4. The solution was then neutralized to pH 8.5 with 1 N NaOH. HPLC analysis showed the sample to be about 40% decomposed.

Titration of CMP-NeuAc and UMP-NeuAc

A 2 mL solution of CMP-NeuAc (6 mM, 0.012 µmols) was placed on ice. The initial pH was measured to be 8.20. Aliquots

of 1N HCl were then added (2 μL, 2 μmols) by a 100 μL syringe with a clicker attachment. This was done with stirring at 4 °C. The pH was measured after each addition of 1 N HCl to a final pH of 2.47. UMP-NeuAc was titrated in an analogous manner beginning with a 2 mL solution of UMP-NeuAc (5.8 mM, 0.0116 μmols). The initial pH was 8.0 and was titrated to a final pH of 2.40.

CHAPTER 3
KINETIC ISOTOPE EFFECTS ON THE ACID CATALYZED SOLVOLYSIS OF SIALYLTRANSFERASE SUBSTRATES

Introduction

With the long term goal of this project being to design transition-state analogs to inhibit sialyltransferases, it is advantageous to begin with a clear picture of what the transition-state structure of the chemical hydrolysis of the donor substrate (CMP-NeuAc) is. While it probably won't be identical to that of the enzyme catalyzed reaction, it is likely that there are similarities and it serves as a good point of comparison. Kinetic isotope effects (KIEs) are a technique perfectly suited for gaining transition-state information about a reaction. By measuring a family of kinetic isotope effects around the reaction center one can gain useful information about the reaction mechanism. By knowing how the substrates behave chemically, one can then gain insight into what roles the catalytic groups in the enzyme active-site may need to play. Comparison of the KIEs measured on chemistry and the enzyme

catalyzed reaction can also give information on how rate determining the chemistry is in the enzyme reaction. This type of approach has been used to probe the mechanism of acid catalyzed hydrolysis of the sialyl donor substrate CMP-NeuAc (86).

Kinetic Isotope Effect Background

Primary ^{14}C Kinetic Isotope Effects

The primary ^{14}C KIE is a way to probe the nature of bond breakage to the isotopically substituted carbon atom. For an associative S_N2 process, values fall in the range of 1.08-1.15. For a dissociative S_N1 mechanism, the effect is in the range of 1.02-1.05 (101). The magnitude of these effects is dependant upon the symmetry of the transition-state. As previously discussed, the isotope effect results from the change in force constants in going from the ground-state to the transition-state. In a reaction that is symmetrical with respect to the isotopically substituted atom, the mass of the isotopically substituted atom will not contribute significantly to the transition-state vibrational frequency. This is because it is not in motion with

respect to the atoms it is being transferred to and from. This results in a situation where the heavy atom and light atom will have very similar vibrational frequencies in the transition-state (67). This occurs in S_N2 reactions which are characterized by larger primary ^{14}C isotope effects.

In the case where the transition-state is unsymmetrical, such as in a dissociative S_N1 mechanism, the isotope effect will be smaller. In this case, the mass of the isotopically substituted atom contributes more because of the unsymmetrical nature of the transition-state. Part of the contribution by the zero-point energy ground-state vibrational frequency will be canceled out reducing the magnitude of the isotope effect (67).

Secondary Kinetic Isotope Effects

A secondary isotope effect is reported on by isotopic substitution at a bond not being broken in the reaction. These effects result from a change in force constant in going from the ground-state to the transition-state. Normal ($k_L/k_H>1$) and inverse ($k_L/k_H<1$) effects are known (L=light and H=heavy). A

normal effect is seen if the lighter molecule has a lower activation barrier to go over than the heavy molecule. This is seen when the isotopically substituted atom is in a looser bonding environment in the transition-state than in the ground-state (101). This situation is illustrated in Figure 3-1.

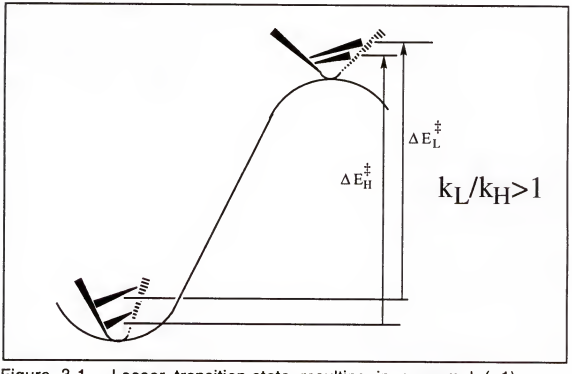


Figure 3-1. Looser transition-state resulting in a normal (>1) isotope effect (Taken from reference 67).

The transition-state energy well is wider indicating a looser bonding environment. Conversely, if the substituted atom is in a tighter bonding environment in the transition-state, an

inverse effect will be measured. This results from the lighter molecule having a higher activation barrier to pass over than the heavier molecule (Figure 3-2). The tighter bonding environment in the transition-state is indicated by the narrower energy well. In this situation, the lighter molecule has a higher activation barrier to pass over and will react slower. A "looser" well has

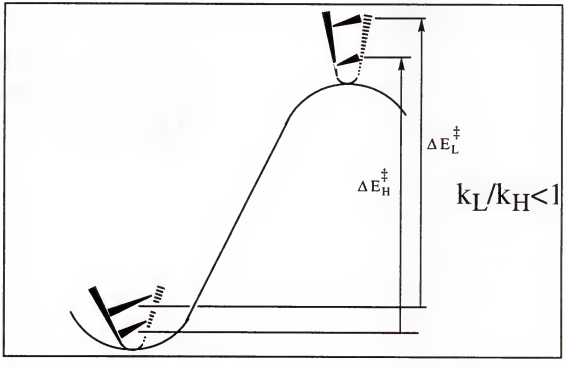


Figure 3-2. Tighter transition-state resulting in an inverse (<1) isotope effect (67).

closer spacing of the zero point energies for the heavy/light isotope sensitive vibrations resulting in $\Delta E^{\ddagger}$(light) being smaller than $\Delta E^{\ddagger}$(heavy) (67).

One specific type of secondary effect is the β-dideuterium effect. This effect is measured by substituting two deuterium atoms β to the bond being broken. The majority of this effect arises from hyperconjugation between the C-H(D) bond and the electron deficient p-orbital (102). This results in a loosening of the C-H(D) bond in the transition-state giving a normal isotope effect as shown in Figure 3-1. The magnitude of the effect depends on the amount of positive charge buildup in the transition-state as well as the dihedral angle of the C-H(D) bond (101, 103) and the electron deficient p-orbital. The dihedral angle determines how well hyperconjugation can occur with maximal effects seen for angles of 0 and 180°. There is also a small conformationally independent inverse inductive effect associated with β-deuterium effects that is believed to result from the bond density of a C-D bond being closer to the carbon than in the C-H bond (104). The β-dideuterium effect is an

excellent way to measure how much positive charge is present at the transition-state of a reaction, and due to the angular dependence (101, 103) this isotope effect can also provide conformational information. These values are typically around 1.1 for a single deuterium with maximum values of 1.4 (105).

Kinetic Isotope Effect Methodology

The procedure for measuring the isotope effects involved isolating unreacted substrate before the reaction was initiated and again when the reaction was near 50% completion. This was done by HPLC (MonoQ anion exchange column) with typical chromatograms as shown in Figure 3.3. The entire substrate peak was collected directly into scintillation vials for counting.

Results

The kinetic isotope effects measured for the solvolysis of CMP-NeuAc and UMP-NeuAc are listed in Table 3-1. For CMP-NeuAc, β-dideuterium effects of 1.276 ± 0.008 at pH 5 and 1.416 ± 0.015 at pH 7 were measured. A β-deuterium effect of 1.112 ±

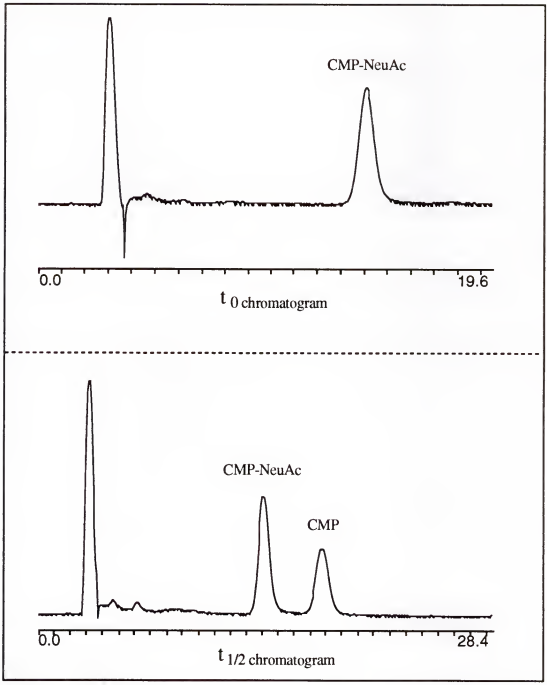


Figure 3-3. Typical KIE HPLC chromatograms. Entire CMP-NeuAc peaks were collected.

0.006 with pro-R CMP-NeuAc was measured to probe the transition-state ring conformation.

Isotopomeric Pair	Type of KIE	Value
[9-^{3}H; 3,3'-$^{2}H_2$]; [^{14}C-N-acetyl] CMP-NeuAc	β-dideuterio	1.276 ± 0.008
[9-^{3}H; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-dideuterio (pH 7)	1.416 ± 0.015
[2-^{14}C]; [9-^{3}H] CMP-NeuAc	primary ^{14}C	1.030 ± 0.005
[1-^{14}C]; [9-^{3}H] CMP-NeuAc	secondary ^{14}C	1.013 ± 0.004
[9-^{3}H]; [1-^{14}C-N-acetyl] CMP-NeuAc	control	1.002 ± 0.010
[9-^{3}H; pro R-^{2}H]; [^{14}C-N-acetyl] CMP-NeuAc	β-deuterio (pro R)	1.112 ± 0.006
[^{3}H-N-acetyl]; [^{14}C-N-acetyl; 2-^{18}O] CMP-NeuAc	^{18}O leaving group	1.003 ± 0.005
[9-^{3}H; 3,3'-$^{2}H_2$]; [^{14}C-N-acetyl] UMP-NeuAc	β-dideuterio	1.28 ± 0.01
[2-^{14}C]; [9-^{3}H] UMP-NeuAc	primary ^{14}C	1.028 ± 0.008

Table 3-1. Solvolysis KIEs. All KIEs were performed using the competitive method at pH 5, 37 °C, unless otherwise indicated.

A primary ^{14}C effect of 1.030 ± 0.005 at the anomeric carbon and a secondary ^{14}C effect of 1.013 ± 0.004 at the

carboxylate were measured with CMP-NeuAc at pH 5. A leaving group ^{18}O effect of 1.003 ± 0.005 was observed with CMP-NeuAc at pH 5. A control experiment with both trace labels in remote positions was performed with an observed isotope effect of 1.002 ± 0.010. To ensure that UMP-NeuAc behaved similarly to CMP-NeuAc with respect to solution chemistry, selected isotope effects were measured on the solvolysis of the slow substrate. A β-dideuterium effect of 1.28 ± 0.01 was measured with UMP-NeuAc at pH 5. A primary ^{14}C effect of 1.028 ± 0.008 was also measured with UMP-NeuAc at pH 5. Both of these effects are well within experimental error of the KIEs measured with CMP-NeuAc.

Discussion

CMP-NeuAc Acid Catalyzed Solvolysis Kinetic Isotope Effects

A primary ^{14}C KIE of 1.030 ± 0.005 was observed for the solvolysis of CMP-NeuAc at pH 5 (86). This value is well below the range of 1.08-1.15 that is expected for an associative S_N2 process which rules out mechanisms involving significant bond order to both the incoming nucleophile and departing leaving

group (101). The observed value is in the range expected for dissociative S_N1 processes. Acid-catalyzed hydrolysis of glycosides give primary ^{14}C isotope effects in the range of 1.02-1.05, corresponding to transition-states with oxocarbenium ion character (106, 107, 108).

A β-dideuterium effect of 1.276 ± 0.008 was measured for the acid-catalyzed solvolysis reaction of CMP-NeuAc at pH 5 (86). This indicates that a large amount of positive charge is present at the reaction center in the transition-state. This value is well within the range expected for β-deuterium effects in carbenium ion-forming reactions and fits well with the primary ^{14}C result (106). Both the dideuterium and primary ^{14}C KIEs show that the solvolysis transition-state has considerable oxocarbenium ion character.

To further probe the nature of the transition-state, a β-deuterium isotope effect was measured on the monodeuterated substrate pro-R CMP-NeuAc. This compound has the axial C3 proton specifically replaced with deuterium. As discussed above,

there is a conformational dependance on the β-deuterium KIE (105). Thus, if the effect from a single deuterium is measured, the dihedral angle may be calculated with knowledge or reasonable estimates of KIE_{max} and $KIE_{inductive}$. The relationship between the effect and the angle is described in eq. 3.1 (105).

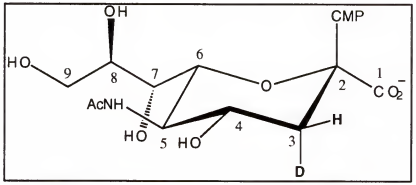


Figure 3-4. Pro-R CMP-NeuAc.

$$\ln KIE_{observed} = \cos^2\theta * (\ln KIE_{maximum}) + \ln KIE_{inductive} \quad \text{eq. 3.1}$$

Because the inductive effect is small (0-2%) (103) and is conformationally independent, it can be ignored when determining θ (109). Therefore, with an estimation of the maximum KIE ($KIE_{maximum}$) along with the KIE for a single deuterium ($KIE_{observed}$),

the angle may be solved for allowing the ring conformation to be estimated.

A β–deuterium KIE of 1.112 ± 0.006 was measured for pro-R CMP-NeuAc. Because secondary isotope effects occurring on the same reaction are multiplicative, the effect on the pro-S can be calculated to be 1.147 (109). Qualitatively, this result suggests that the pro-S proton is able to hyperconjugate better with the electron deficient p-orbital. To determine θ an estimate of KIE_{max} is needed. A value for KIE_{max} 1.4 is a reasonable estimate, paralleling Sinnott's treatment of this situation in his study of the hydrolysis or aryl glycosides of NeuAc (109). Using this value for KIE_{max} and substituting in 1.112 for $KIE_{observed}$ into eq. 3.1, a dihedral angle of 55° is determined for the pro R proton (this assumes there is no inductive effect as was done by Sinnott). The dihedral angle for the pro-S proton is then 175° (θ+120°). These angles are referenced to the anomeric carbon-leaving group bond being set at 0°. This would result in a transition-state structure as shown below in Figure 3-5. The ring becomes flattened out as a result of the formation of the oxocarbenium ion with the C3

protons positioned to allow the pro-S proton to overlap better with the electron vacant p-orbital of the anomeric carbon. Because KIE_{max} is an estimate, the calculated angles are only as good as its estimation, and should only by used qualitatively. As illustrated in Figure 3-5 the leaving group (CMP) is also very distant from the reaction center in the transition-state and a nearly full charge is present at the anomeric carbon. Additionally, these results indicate no nucleophilic participation aiding in leaving group departure (86, 90).

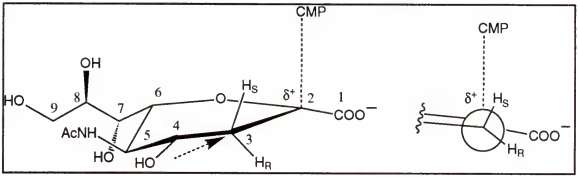


Figure 3-5. Transition-state structure of the solvolysis reaction. Dashed arrow represents Newman projection view down C3-C2 bond.

A mechanistically interesting feature of NeuAc is the presence of the carboxylate located next to the reaction center.

While it is believed that this group plays a major role in the biological activity of NeuAc (2), its role in catalysis of reactions involving this compound are only now becoming clear (91). Initially, the carboxylate carbon of NeuAc was envisioned to be ideal location for a trace label. However, an unexpected ^{14}C isotope effect of 1.013 ± 0.004 was measured at this position. This value is unusually large for a secondary heavy atom isotope effect (101). A normal isotope effect of this magnitude indicates a loosening of the carboxylate upon reaching the transition-state. One mechanistic possibility to account for this result is that the carboxylate O-C-O bond angle opens up upon formation of the oxocarbenium ion. This would occur by the electron density of the carboxylate oxygens being pulled back towards the anomeric carbon in order to provide electrostatic stabilization of the oxocarbenium ion. Such a transition-state would result in the carboxylate carbon being in a looser environment (86). It has been estimated that the carboxylate may provide 17 kcal/mol of stabilization to the oxocarbenium ion (91).

To further characterize the transition-state structure around the anomeric carbon, we sought to probe the nature of the leaving group bond during the reaction. This was done by measuring a primary ^{18}O isotope effect on the leaving group oxygen. The CMP-NeuAc isotopomers used in this experiment were [^{3}H-NAc] CMP-NeuAc and [1-^{14}C-NAc; 2-^{18}O] CMP-NeuAc. The amount of ^{18}O present in the latter compound was determined by integration of the peaks of the ^{16}O and ^{18}O species in the proton decoupled ^{31}P-NMR. A leaving group ^{18}O KIE of 1.003 ± 0.005 was observed. Unfortunately, this unity KIE is not diagnostic. For a dissociative type mechanism in which the leaving group bond is significantly broken in the transition-state, primary ^{18}O effects can be as much as a few percent (110). Complexity can arise in interpretation of this type of effect, however, due to the likelihood of a specific acid catalyst participating in leaving group departure. Thus, even though the leaving group bond to the anomeric carbon is significantly broken, much of this lost bond order can be made up for by the transferred

proton. This would result in the isotopically substituted oxygen experiencing little overall change in bond order in going from the ground-state to the transition-state (111). The above considerations include an assumption that the labeled bridging oxygen is the site of protonation. This issue is presently being examined in the Horenstein lab via computational approaches.

UMP-NeuAc Acid Catalyzed Solvolysis Kinetic Isotope Effects

In order to allow proper interpretation of kinetic isotope effects measured with the UMP-NeuAc isotopomers for sialyltransferase reactions, it was first necessary to confirm that substitution of a keto group for an amino group on the pyrimidine ring did not alter the KIEs measured on the NeuAc residue. While it was not anticipated that such a remote alteration would have an impact on the chemical properties of the reaction center, controls were performed to support this assumption.

Acid catalyzed solvolysis of UMP-NeuAc at pH 5 afforded an isotope effect of 1.28 ± 0.01, in excellent agreement with the

previously measured value of 1.276 ± 0.008 for CMP-NeuAc. A primary ^{14}C effect of 1.028 ± 0.008 was seen upon acid catalyzed hydrolysis, also agreeing well with the value of 1.030 ± 0.005 measured for CMP-NeuAc. These results indicate that any difference in the magnitude of isotope effects measured on enzyme catalyzed reactions when using UMP-NeuAc, as opposed to CMP-NeuAc, will originate in a change of rate-determining steps and not the chemical nature of the substrate.

Conclusions

The results of the KIEs measured on the acid catalyzed solvolysis of CMP-NeuAc point to a very late transition-state which possesses substantial oxocarbenium ion character and involves little nucleophilic participation. It has also been shown that the transition-state for the solvolysis of UMP-NeuAc is the same as that of CMP-NeuAc. These results are used as a point of reference in the work performed on the sialyltransferase catalyzed reaction.

Experimental

General Solvolysis Kinetic Isotope Effect Methodology

Solvolysis kinetic isotope effect (KIE) experiments used mixtures of radiolabeled isotopomeric substrates, and were therefore competitive (112). Experiments employed ca. 100,000 CPM each of the appropriate ^{3}H- and ^{14}C- labeled CMP-NeuAc isotopomers. For each experiment a master mixture of a given $^{3}H/^{14}C$-labeled isotopomeric pair of CMP-NeuAc was prepared from which aliquots were withdrawn for individual reactions and measurement of the reference $^{3}H/^{14}C$ ratio at time 0. Reaction mixtures (50-500μL, pH 5-7) were initiated by the addition of the appropriate volume of 1 N acetate buffer (pH 5-6) or 80 mM sodium cacodylate (pH 7) to the isotopomeric mixture of CMP-NeuAc and placed at 37 °C. After 40-60% conversion (68), unreacted substrate was isolated from the reaction mixture on a MonoQ HR10/10 column (100 mM NH_4HCO_3, 15% methanol, pH 8.0, 2 mL/min, A_{260}) and collected directly into scintillation vials (2 mL fractions). Care was taken to collect the entire CMP-NeuAc

peak. The percent conversion was determined from the CMP-NeuAc and CMP peaks in the HPLC chromatogram. The initial $^{3}H/^{14}C$ ratio was obtained in triplicate by injecting aliquots of the master mixture of labeled CMP-NeuAc on the MonoQ column and recollecting the entire CMP-NeuAc peak. The $^{3}H/^{14}C$ ratios for CMP-NeuAcs were determined by dual-channel liquid scintillation counting (channel A, 0-15 keV; channel B 15-80 keV) with each tube being counted for 10 min, and all tubes cycled through the counter 6-10 times. The internal ^{133}Ba source was used to estimate the quench parameter for each tube, which varied by ±1%. Triplicate samples of [^{14}C] CMP-NeuAc were used to determine the ratio of ^{14}C counts in channels A and B ($A:B_{14}$). Since ^{3}H is only detected in channel A, the $^{3}H/^{14}C$ ratio in a given tube was calculated with equation 3.2 (112).

$$^{3}H/^{14}C = (\text{cpm A} - \text{cpm B} \times A:B_{14}) / (\text{cpm B} + \text{cpm B} \times A:B_{14}) \quad \text{eq. 3.2}$$

The reported value and error of a KIE represents the mean and standard deviation of three individual KIE experiments taken over 6-10 cycles through the liquid scintillation counter. The following equations were used to calculate the KIEs:

$$\beta\text{-}^{2}H\ KIE_{observed} = (^{3}H/^{14}C)_{t1/2} / (^{3}H/^{14}C)_{0} \qquad \text{eq. 3.3}$$

$$^{14}C\ KIE_{observed} = (^{14}C/^{3}H)_{t1/2} / (^{14}C/^{3}H)_{0} \qquad \text{eq. 3.4}$$

The KIEs were then corrected for % conversion using equation 3.5 (69):

$$KIE_{corrected} = \ln(1-f) / \ln((1-f) \times KIE_{observed}) \qquad \text{eq. 3.5}$$

f = % conversion

For KIEs employing stable isotopes where complete incorporation was not achieved (pro-R and ^{18}O KIEs), the following equation was used to correct the observed KIEs (111):

$$KIE_{corrected} = (KIE_{observed} - 1 + f) / f \qquad \text{eq. 3.6}$$

f = fraction of stable isotope incorporation

The isotope effects on the solvolysis of UMP-NeuAc were carried out in an identical manner with the appropriate UMP-NeuAc isotopomers.

CHAPTER 4
ALPHA (2→6) SIALYLTRANSFERASE KINETIC ISOTOPE EFFECTS

Introduction and Background

Isotope effects are a powerful tool for elucidating reaction mechanisms on both chemical processes as well as enzymatic ones, but when applied to enzyme reactions their interpretation can be considerably more complex. As previously discussed, in order for a kinetic isotope effect on a chemical step to be measurable, that step must be rate-determining, or at least partially so. In enzymatic reactions there are minimally two more necessary steps to be considered. These are binding of substrate to form the Michaelis complex and the subsequent release of product. In addition there may be one or more conformational changes of the protein structure necessary for catalysis that could have rates similar to that of chemistry. With enzymes having evolved to catalyze reactions so well, it is very common for chemistry to be fast relative to these steps. In fact, it has been stated that it is rare for chemistry to be completely rate-limiting in enzymic reactions (113). This serves

to diminish the observed isotope effects by masking the intrinsic effects. Intrinsic isotope effects are those that would be seen if chemistry was completely rate-limiting. These types of complexities are termed commitments to catalysis (114, 116). This is shown in the scheme below where there is a binding step, a conformational change, chemistry, and finally product release. This can provide extreme experimental challenge. The modeling

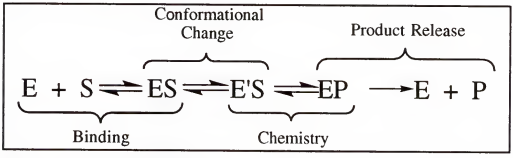


Figure 4-1. Theoretical enzymatic scheme.

of transition-state structures can only be done if the magnitude of intrinsic isotope effects are known. Because the magnitude of an isotope effect can be diagnostic for a particular reaction mechanism, the incorrect assumption that observed isotope effects are intrinsic can lead to erroneous conclusions regarding

charge build-up or bond order in the transition-state. Determining how close observed isotope effects are to intrinsic values can also present a problem. One way to estimate this is to compare the observed values on the enzymatic reactions to those of a comparable chemical reaction. While this approach also has its caveats, it is often a reasonable one because many if not most chemical reactions will not have kinetic complexity. The isotope effect on the chemical reaction represents an upper limit to the size of an isotope effect on the enzyme catalyzed reaction. If it turns out that observed KIEs for the enzyme catalyzed reaction are smaller than expected and it is suspected that chemistry is not completely rate-limiting, suitable conditions for measuring intrinsic effects should be found.

Some reaction conditions which may be tailored to provide intrinsic isotope effects are pH, ionic strength, solvent polarity, or even temperature. An alternative approach is to use a "slow" substrate which has a diminished rate of catalysis (114). The goal of these approaches is to eliminate commitments associated with catalysis.

A commitment for a particular reactant is defined as the ratio of the rate-constant for the isotope-sensitive step to the net rate constant for release of the reactant from the enzyme (114). Observed V/K isotope effects may be corrected for commitments to catalysis by eq. 4.1. This equation is for reactions which are not reversible. The possibility also exists for there to be multiple commitments on different steps in the mechanism, as well as in the forward or reverse direction.

$$KIE_{intrinsic} = KIE_{observed} + (KIE_{observed} * C_f) - C_f \qquad \text{eq. 4.1}$$

Analysis of the hypothetical mechanism below illustrates some of the different types of possible commitments. Figure 4-2 represents the case for a single substrate, and it may be extended

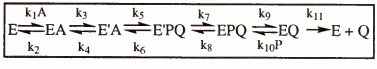


Figure 4-2. Multi-step enzyme mechanism (Taken from reference 114).

to multi-substrate reactions as well. In this example it is assumed that k_5 and k_6 are the only isotopically sensitive steps (this assumption breaks down in some multi-step reactions and when binding effects are present (114). The external commitment is the partition ratio for the initial collision complex EA and is given by k_3k_5/k_2k_4. The internal commitment is the partition ratio for any intermediates, and is k_5/k_4 for A in this mechanism. The combination of the internal and external commitments is the total commitment. Often, the external commitment can be eliminated by altering the pH. The external commitment can also possibly be removed through the use of a slow substrate where k_3k_5/k_4 is less than k_2 even when the pH is at its optimum (114).

While these are reasonable approaches to addressing external commitments, eliminating internal commitments may be more challenging. Internal commitments are usually pH independent. While a slow substrate can eliminate an internal commitment, it will not do so if the decrease in rate is a result

of slower initial isomerization of the Michaelis complex (EA), or a slower rate of product release. If k_5 and k_4 change in parallel the commitment will also not be removed (114).

The previous examples and discussion have focused on the single substrate scenario, and while the basic principles are applicable to bi-substrate systems, the issue of how substrate concentrations may influence commitments will now be addressed. In an ordered bi-substrate mechanism it can be seen that the commitment for the first substrate (A) will be directly proportional to the concentration of the second substrate (B), reaching an infinite value when the second substrate is saturating. Under these conditions, any time that A is bound it will be locked in by B and "committed" to go through the catalytic cycle. Any kinetic isotope effects measured on A at saturating levels of B will be unity.

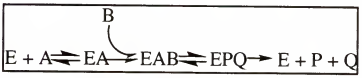


Figure 4-3. Ordered bi-substrate mechanism.

The commitment for B is concentration independent, and is a function of the ratio of the rate for the chemical step to its off-rate for the ternary (EAB) complex. While a similar trend can be postulated for a random bi-substrate mechanism (Figure 4-4), it may not necessarily be as extreme. In this case the dependence of the commitment of one substrate on the concentration of the other substrate will depend on the on and off rate constants for each substrate in the ternary complex (EAB) in addition to concentration (113). For example, if A* were the isotope-labeled substrate, at saturating levels of B, EA* would be driven towards EA*B. Even at saturating levels of B, A* would still have an escape route from EA*B because the mechanism is random. Since saturating B is unable to completely trap A* on EA*B, the commitment is finite and will depend on the ratio of the rate constant for the catalytic step to the rate constant for release of A* from EA*B. The step(s) that the isotope effect is reporting on in an enzymatic reaction may also be dependent on substrate concentration. This phenomena has to do with the saturation level of the enzyme. While the rates of all enzymatic reactions

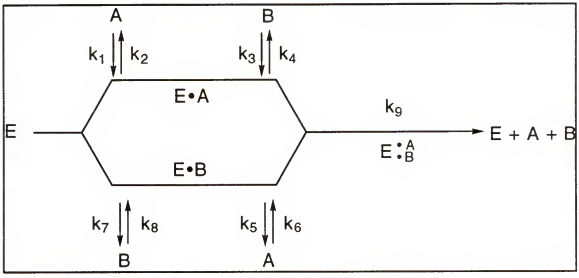


Figure 4-4. Random bi-substrate mechanism.

are proportional to the amount of enzyme present, substrate concentration will determine which steps in the reaction are rate-limiting. Conditions under which substrate concentrations are subsaturating are denoted V_{max}/K_m, which is an apparent second order rate constant for combination of enzyme and substrate. The physical meaning of this is that the rate of the reaction is dependent on both the maximal rate of catalysis (V_{max}) as well as the substrate binding constant (K_m). When there is a saturating amount of substrate present, the conditions are

described as V_{max}. In this situation the rate of the reaction is only dependent upon the maximal rate of catalysis by the enzyme and not substrate concentration. Depending on which conditions an experiment is run under, different steps in the catalytic cycle will be reported on. Under V/K conditions the steps under examination will be all those up to and including the first irreversible step. Usually the first irreversible step will be the first chemistry step, but this is not always the case. When there is a saturating level of substrate (V_{max} conditions) all steps in the mechanism after binding and through product release which are rate-determining will be reported on. While an isotope effect measured under either conditions may give the same result, it is common for V/K and V_{max} effects to have different values. This can give information on the identity of rate determining steps in the mechanism (114).

This chapter describes our approach to determining the external commitment to catalysis through an isotope trapping experiment and the correction of the kinetic isotope effects measured with CMP-NeuAc using the experimentally determined

value for the external commitment. Also included is how we have eliminated the internal commitment to catalysis through the measurement of kinetic isotope effects with the "slow" substrate UMP-NeuAc.

Results

An isotope trapping experiment was conducted to investigate the partitioning of the initial Michaelis complex (E:CMP-NeuAc) between dissociation of substrate and product formation. The results are shown below in Figure 4-5. The data points represent the amount of radiolabeled product formed at a particular concentration of the second substrate LacNAc and is plotted in double reciprocal form. With this experiment we determined the external commitment to catalysis for $\alpha(2\rightarrow6)$ sialyltransferase to be 1 and 1.7 at 1.6 mM and 100 mM LacNAc, respectively. As shown in Figure 4-5, a finite amount of CMP-NeuAc was trapped on the enzyme, but saturation with LacNAc did not trap all of the bound CMP-NeuAc as product.

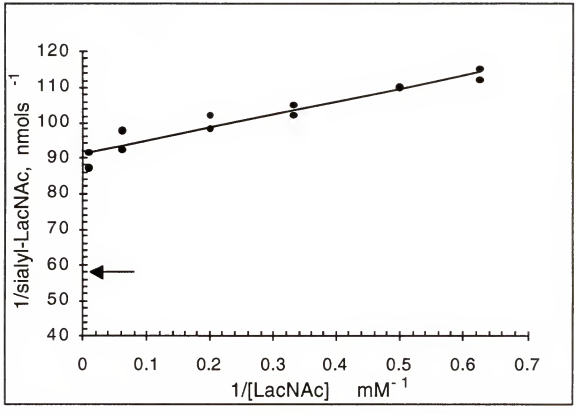


Figure 4-5. Isotope trapping experiment. Arrow indicates the amount of product expected for complete trapping of CMP-NeuAc.

The KIEs measured for $\alpha(2\rightarrow6)$ sialyltransferase (86) are listed in Table 4-1. These isotope effects have been corrected using our experimentally determined value for the external commitment (1 at 1.6 mM LacNAc) and eq. 4.1. These isotope effects were measured under V/K conditions using the competitive method. The acceptor substrate N-acetyllactosamine

Entry	Isotopomeric Pair	Type of KIE	Value
1	[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-secondary	1.044 ± 0.007
2	[9-^{3}H; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-secondary	1.066 ± 0.007
3	[9-^{3}H; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-secondary (100 mM LacNAc)	1.068 ± 0.007
4	[2-^{14}C]; [1-^{3}H-N-acetyl] CMP-NeuAc	primary ^{14}C	1.000 ± 0.004
5	[1-^{14}C]; [1-^{3}H-N-acetyl] CMP-NeuAc	secondary ^{14}C	0.998 ± 0.004
6	[9-^{3}H]; [1-^{14}C-N-acetyl] CMP-NeuAc	binding	0.984 ± 0.007
7	[9-^{3}H; pro-R ^{2}H]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-deuterio (pro-R)	1.010 ± 0.003
8	[1-^{3}H-N-acetyl]; [1-^{14}C-N-acetyl; 2-^{18}O] CMP-NeuAc	^{18}O leaving group	0.997 ± 0.006
9	[1-^{3}H-N-acetyl]; [1-^{14}C-N-acetyl] CMP-NeuAc	control	1.003 ± 0.004
10	[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-secondary (pH 8)	1.008 ± 0.004
11	[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc	β-secondary (pH 5)	1.117 ± 0.011
12	[2-^{14}C]; [9-^{3}H] CMP-NeuAc	primary ^{14}C (pH 5)	1.019 ± 0.012
13	[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] UMP-NeuAc	β-secondary	1.218 ± 0.010
14	[9-^{3}H]; [1-^{14}C-N-acetyl] UMP-NeuAc	binding	0.944 ± 0.010
15	[2-^{14}C]; [1-^{3}H-N-acetyl] UMP-NeuAc	primary ^{14}C	1.028 ± 0.010
16	[1-^{3}H-N-acetyl]; [1-^{14}C-N-acetyl] UMP-NeuAc	control	1.005 ± 0.005

Table 4-1. KIEs on α(2→6) sialyltransferase reaction.

(LacNAc) was used in these experiments at its K_m value (1.6 mM), unless otherwise indicated, and the effects measured on UMP-NeuAc were done with a LacNAc concentration of 40 mM. The observed effects on CMP-NeuAc at pH 7 were then corrected using our experimentally determined value for the external commitment to catalysis using eq. 4.1. The reported KIEs with CMP-NeuAc as the donor substrate were unusual. The β-dideuterium KIEs were very small relative to those measured for solvolysis. Additionally, we could not readily explain the measurement of a unity primary ^{14}C isotope effect at the anomeric carbon. These results suggested that either the reaction mechanism for sialyltransferase proceeds through a very different transition-state than that for solvolysis, or there is kinetic complexity (commitments to catalysis) associated with the sialyltransferase reaction which is masking the isotope effects. When the β-dideuterium effects were measured at lower pH, the observed effects increased. This indicated that commitments to catalysis were being removed and suggested that the small

isotope effects with CMP-NeuAc were due to kinetic complexity. To further address the issue of removing commitments, the isotope effects with the alternative substrate UMP-NeuAc were measured. The isotope effects measured with UMP-NeuAc indicate that it is a "slow" substrate and does not have any significant commitments associated with it. A detailed discussion of the observed measured isotope effects and their interpretations follows.

Discussion

Isotope partition experiment

An informative kinetic experiment is an isotope partition study (115). The experimental procedure utilizes radiolabeled substrate in a pulse-chase fashion. In its most general form, isotope partition experiments report on the relative rate that bound labeled substrate leads to product compared to the rate of other steps that the bound labeled substrate can undergo (115). This type of information is particularly valuable to help interpret observed kinetic isotope effects, because the extent that

chemistry is rate-determining must be known in order to correctly interpret the observed isotope effects (116). This experiment can also help to answer the questions of when substrates combine and products depart in the mechanism, as well as if there are covalent intermediates involved.

The use of the isotope trapping experiment was first applied to study the partitioning of a noncovalent Michaelis complex in the case of yeast hexokinase and glucose (117). In this instance the glucose in the enzyme-glucose complex was able to be trapped and shown to be completely locked in the active site relative to the rate of product formation. This is an example of how isotope trapping can give both qualitative and quantitative data. Isotope trapping can give evidence to help establish if a mechanism is random or ordered. In a steady-state ordered mechanism, all of the substrate that binds to the enzyme first should be trapped when exposed to a saturating amount of the second substrate. Conversely, if the enzyme was pre-incubated with radiolabeled substrate that binds second, no radiolabeled product should be seen, since the binary complex

cannot form. In a random mechanism all of the enzyme bound substrate would not necessarily be trapped. The failure to trap any or less than all of the E*A complex can result from three scenarios. One is that there may be more than one species of complex present, some of which are not competent Michaelis complexes. A second is that the dissociation rate of *A from E*A is too rapid even at $[B]>K_m$ for *A to be trapped. Finally, *A could dissociate from E*AB at a rate greater than V_{max} (115).

The quantitative information this experiment can provide is the amount of substrate that is bound properly, meaning that the substrate does not have to be released before the catalytic cycle can occur. This information is related to the commitment factor, a kinetic parameter that describes the commitment of the Michaelis complex to undergo chemistry. This parameter can be a measure of how rate determining chemistry is in the catalytic mechanism and may be used to calculate intrinsic isotope effects from the observed effects by using eq. 4.1 (114). Intrinsic values are those that would be observed if chemistry was completely rate limiting. If there is a significant commitment to catalysis,

meaning binding of the substrate is at least partially rate limiting, the magnitude of the isotope effect will be diminished. Care must be taken in this approach, however, because the mechanism and experimental conditions can influence the relationship between isotope trapping and commitment factors.

The isotope trapping ratio obtained from the trapping experiment is conceptually equivalent to the commitment to catalysis, but the two are not necessarily equivalent (118, 119). This is because the isotope trapping ratio is a description of what occurs under single turnover conditions whereas the commitment to catalysis is descriptive of what occurs under steady state conditions where the enzyme can bind and dissociate the labeled substrate many times during the reaction. In a random mechanism, the isotope trapping ratio determined at saturating levels of the second substrate will closely approximate C_f. This is because the initial binary complex is driven to the ternary complex when the second substrate is saturating at which point two events could occur: dissociation of the labeled substrate or product formation (trapping). The

observed isotope trapping is dependent only upon the ratio of the rates of these two events. The value of C_f is also dependent upon the same rate constants and is thus directly given under the conditions where the second substrate is saturating. Also, the isotope trapping ratio may still closely approximate C_f even if the second substrate is not saturating as long as the off-rate of the labeled substrate in the binary complex is slow relative to the on-rate of the second substrate. If the off-rate of the labeled substrate in the binary complex is faster than the formation of the ternary complex, the isotope trapping will underestimate the true value of C_f (119).

The isotope trapping data of interest for the present study is that of the donor substrate CMP-NeuAc. The commitment factor for this substrate is needed because this is the substrate that has been used in many of the kinetic isotope effect experiments, and prior to the present study there were ambiguities regarding the kinetic mechanism for sialyltransferase. Our experimental approach was a variation of the Rose method. The experimental design utilized the

competitive inhibitor cytidine-5'-diphosphate (CDP) (120) in the chase solution instead of excess unlabeled substrate. This compound was more readily available and is bound by the enzyme approximately as tightly as CMP-NeuAc. Control experiments demonstrated that the competitive inhibitor was an effective quench for the enzyme over the time course of the experiment. In this approach the enzyme was pre-incubated with a saturating amount of radiolabeled CMP-NeuAc with a known specific activity before being placed into a larger volume of reaction mixture containing various concentrations of LacNAc and fixed, saturating amounts of CDP. Under these conditions the saturating amounts of CDP ensure that if radiolabeled CMP-NeuAc dissociates, it is replaced by CDP, thus resulting in single turnover conditions. The experiment is outlined in Figure 4-6. The isotope trapping ratio is expressed as shown in eq. 4.2. In this equation [P*] is the amount of radiolabeled sialyl-LacNAc formed in a single turnover while the denominator represents the amount of bound labeled CMP-NeuAc which dissociated from the enzyme. The $\alpha(2\rightarrow6)$ sialyltransferase kinetic mechanism is shown in Figure 4-7.

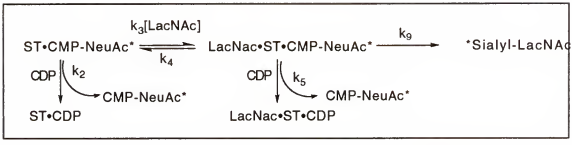


Figure 4-6. Isotope trapping with competitive inhibitor.

$$\text{trapping ratio} = [P^*]/([E_T]-[P^*]) \qquad \text{eq. 4.2}$$

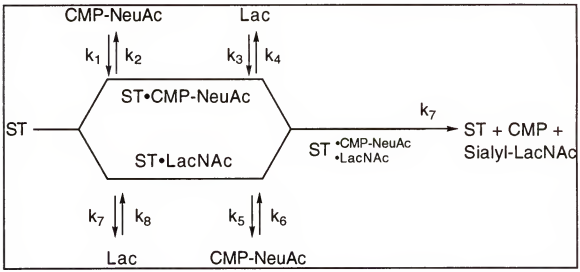


Figure 4-7. Random kinetic mechanism of $\alpha(2\rightarrow6)$ sialyltransferase. Rate constant k_7 includes all steps after substrate binding.

For a random kinetic mechanism the isotope partition experiment is described by eq. 4.3 (121). The collection of rate constants which make up the steady-state commitment to catalysis for V/K are given by eq. 4.4 (122). C_f is then related to the isotope trapping ratio by eq. 4.5 (119). Examination of eq. 4.5 illustrates that if the rate at which enzyme bound labeled substrate proceeds to the ternary complex ($k_3[B]$) is much faster than the rate of dissociation of the binary complex (k_2), the isotope trapping ratio directly provides C_f as eq. 4.5 reduces to eq. 4.2 (119).

$$[P^*] / [E_T] = k_3k_5[B] / [k_2(k_4 + k_5 + k_7) + [k_3(k_5 + k_7)[B]]] \quad \text{eq. 4.3}$$

$$C_f = k_5/[k_7 + [k_2k_4/(k_2 + k_3[B])]] \quad \text{eq. 4.4}$$

$$C_f = [P^*][(k_2/k_3[B]) + 1] / [[E_T] - [P^*][(k_2/k_3[B]) + 1]] \quad \text{eq. 4.5}$$

The results of the isotope trapping experiment are shown in Figure 4-5. The data is a plotted in double reciprocal form. The ordinate represents the reciprocal of the amount of product formed, while the abscissa represents the reciprocal of the

concentration of LacNAc used. The experiment was performed in duplicate with different batches of enzyme and substrate to demonstrate reproducibility. The resulting trapping ratios at 1.6 mM and 100 mM LacNAc were 1.0 and 1.7, respectively (119). From the isotope trapping data the off-rate for labeled CMP-NeuAc from the binary complex (k_2) can be estimated to be 3-4.7 s^{-1} (121), and typical values for protein-small molecule association rates are 10^6-10^8 $M^{-1}s^{-1}$ (123). The substitution of these rate constants into eq. 4.5 shows that the isotope trapping ratios are insignificantly lower than the commitment to catalysis, and thus can be used directly as commitments to correct the observed isotope effects.

The isotope trapping experiment has provided both qualitative and quantitative information on the mechanism of $\alpha(2\rightarrow6)$ sialyltransferase. Qualitatively, the kinetics have been shown to be steady-state since a rapid equilibrium mechanism would have substrate off-rates too high to allow trapping of labeled product (120). Strong evidence is also provided for a random mechanism. If the mechanism were strictly ordered with

CMP-NeuAc binding first, all of it would have been trapped at saturating LacNAc. If LacNAc were bound first in an ordered mechanism, none of the initial E:CMP-NeuAc* complex would have been trapped. Quantitatively, we have measured an isotope trapping ratio which has been shown to be equivalent to the commitment to catalysis, allowing the observed isotope effects for $\alpha(2\rightarrow6)$ sialyltransferase with CMP-NeuAc to be corrected for the external commitment to catalysis.

KIEs for $\alpha(2\rightarrow6)$ Sialyltransferase with CMP-NeuAc

A β-dideuterium KIE of 1.033 ± 0.007 was observed using the isotopomer pair of [9-^{3}H; 3,3'-^{2}H] and [^{14}C-NAc] CMP-NeuAc. This observed KIE was corrected for external commitment to give a KIE of 1.066 ± 0.007 using the commitment factor and eq. 4.1. To ensure that the remote radiolabels used in this experiment were "silent" with respect to an isotope effect, a control KIE was measured using [9-^{3}H] and [^{14}C-NAc] CMP-NeuAc. An inverse effect of 0.992 ± 0.007 was observed. This observed effect was

corrected to 0.984 ± 0.007 using the commitment factor. While this effect is very close to unity, it indicates that there is a small binding effect associated with the 9-^{3}H label in the polyhydroxyl tail. The fact that it is inverse indicates that the tritium label is in a tighter bonding environment when enzyme bound. This would be expected if the polyhydroxyl tail was experiencing a binding interaction with the enzyme active-site. In order to measure isotope effects without the presence of the binding effect, these experiments were repeated with the ^{3}H label moved to the N-acetyl portion of the molecule. An isotope effect of 1.022 ±0.007 was measured with the mixture consisting of [^{3}H-NAc;3,3'-^{2}H] and [^{14}C-NAc] CMP-NeuAc. This observed effect becomes 1.044 ± 0.007 when corrected for the external commitment to catalysis. This result indicates that the binding effect is no longer present with the label moved to the N-acetyl position. This conclusion was confirmed when a unity KIE of 1.003 ± 0.004 was measured for the isotopomer pair of [^{3}H-NAc] and [^{14}C-NAc] CMP-NeuAc. Clearly the β-dideuterium effect on

the enzyme reaction is significantly smaller in magnitude than that of the solvolysis reaction. One explanation for this result is simply that the transition-state of the enzyme catalyzed reaction does not have the extensive oxocarbenium ion character found for solvolysis of CMP-NeuAc. This β-dideuterium effect is almost identical the those measured (1.058 ± 0.019) on the hydrolysis of p-nitrophenyl glycoside of NeuAc by neuraminidase from *Vibrio cholerae* (124). The low value for this effect was attributed to nucleophilic participation of the carboxylate group. We do not consider this occurrence likely in the sialyltransferase reaction due to the inverting nature of this enzyme. An alternate explanation for the small effect is that the conformation of the transition-state of the enzyme catalyzed reaction is less than ideal for hyperconjugation. As previously discussed, the magnitude of the effect has an angular dependence with the hyperconjugation of the C-H bonds and the electron deficient p-orbital will be a maximum at 0° or 180°, and a minimum at 90°. However, this hypothesis is readily dismissed by the calculations outlined by Sinnott. In order for observed effects this small to be

observed, it is necessary to begin with a much smaller KIE $_{max}$ in eq. 3.3 (109). This would indicate that there is less than full charge development at the anomeric carbon upon reaching the transition-state which would be indicative of an early transition-state. A final and less easily addressed explanation for the low effects is a large internal commitment to catalysis not measurable by the isotope trapping experiment. This could occur if a slow conformational change occurs in the catalytic mechanism. This issue will be addressed in the discussion of the isotope effects measured on the slow substrate UMP-NeuAc.

An observed isotope effect of 1.000 ± 0.004 was measured for the isotopomer pair of [^{3}H-NAc] and [2-^{14}C] CMP-NeuAc. A unity primary ^{14}C KIE was quite unexpected for the enzyme catalyzed reaction. Solvolysis of CMP-NeuAc proceeded with a primary ^{14}C KIE of 1.030, a typical value for a dissociative S_N1 mechanism. Any bond cleavage at the anomeric carbon should have a normal contribution to the isotope effect due to the reaction coordinate motion which should result in a small (1.02-1.05) normal KIE (101). An explanation for this result is the

possibility of the binding of CMP-NeuAc having an inverse binding isotope effect associated with it. A small inverse binding effect could offset a small normal effect associated with bond cleavage resulting in an observed unity effect. Support for this hypothesis is found in *ab initio* calculations, but has not been experimentally addressed as of yet (119). Alternatively, the unity effect could be explained by an internal commitment to catalysis. It is possible that the unity effect is actually an extremely small normal effect being almost entirely masked by the internal commitment.

With one of the goals of this project being to probe the role of the carboxylate in catalysis, a secondary ^{14}C effect at the carboxylate was measured. The isotopomer pair [^{3}H-NAc] and [1-^{14}C] CMP-NeuAc was used for these experiments. A unity KIE of 0.999 ± 0.004 was observed compared to the fairly large KIE of 1.013 ± 0.004 on solvolysis. The unity KIE on the enzyme reaction at this position could result from no change in bonding environment of the carboxylate due to little charge development

at the reaction center, or the effect could be masked by commitments to catalysis.

To probe the cleavage of the leaving group bond further, a leaving group ^{18}O effect was measured. The isotopomeric substrate pair for this experiment was [^{3}H-NAc] CMP-NeuAc and [^{14}C-NAc;2-^{18}O] CMP-NeuAc. A unity KIE of 0.997 ± 0.006 was measured at this position. This result is not very diagnostic. It could be due to the masking of an effect by a commitment, however, it would also be consistent with substantial bond cleavage aided by a general acid catalyst in the active site. In this case the bonding of the proton to the leaving group oxygen would make up for lost bond order with the anomeric carbon upon bond cleavage. Thus, the force constant to the leaving group oxygen would not change dramatically upon reaching the transition-state.

Although the family of observed KIEs measured for $\alpha(2\rightarrow6)$ sialyltransferase were corrected for the external commitment, the large discrepancy with the solvolysis isotope effects was bothersome. It seemed very likely that these results were due to

the presence of a large internal commitment to catalysis. To explore this possibility, the simplest approach was taken initially. The β-dideuterium isotope effects were measured at altered pH and ionic strength in an attempt to eliminate commitments.

The first experiment was performed under more alkaline conditions. The β-dideuterio isotope effect was measured at pH 8 using the isotopomer pair of [^{3}H-NAc; 3,3'-D_2] and [^{14}C-NAc] CMP-NeuAc. Because the pH-rate profile indicated a decrease in rate at this pH, it was believed that chemistry would be slowed down relative to other steps, resulting in observed KIEs closer to intrinsic values. However, an effect of 1.008 ± 0.004 was observed indicating that chemistry is even less rate-limiting at pH 8. This observation led to a shift in strategy in which KIEs would be measured under acidic conditions. Performing kinetic experiments with CMP-NeuAc under acidic conditions brings about the additional concern of background hydrolysis which necessitates stringent controls. The first acidic KIE was run at pH 6 using the same isotopomer pair as the effect measured at pH

8. The observed value was 1.037 ± 0.006 (compared to 1.022 at pH 7). A control reaction indicated that no background hydrolysis occurred over the time period of the experiment. While this observed effect is still much smaller than that for hydrolysis, it is almost twice as large as the value obtained at pH 7. With this indication that a commitment was being removed, the experiment was repeated at pH 5. Under these conditions the corrected observed isotope effect was increased to a value of 1.117 ± 0.011. A control experiment revealed that 5% background hydrolysis took place in the course of the reaction, however, so the observed KIE was corrected for this. When two reaction pathways contribute to an effect, the observed effect is a weighted average of the two and is given by eq. 4.6 (86) shown below.

$$KIE_{observed} = (KIE_1 * KIE_2) / [(KIE_1 * f_2) + (KIE_2 * f_1)] \quad \text{eq. 4.6}$$

In this equation $KIE_{observed}$ is the actual isotope effect measured, KIE_1 and KIE_2 are the effects for the two

simultaneously occurring pathways, and f_1 and f_2 are the fraction of the total conversion coming from each pathway. From this equation and the information obtained, the isotope effect on the enzyme catalyzed reaction at pH 5 can be calculated. The $KIE_{observed}$ is 1.117, the KIE for acid catalyzed hydrolysis at pH 5 is 1.28 and is designated as KIE_1 (Chapter 3, Table 3-1). The values for f_1 and f_2 are known from the HPLC chromatograms of the enzyme reaction and control reaction. Thus, the isotope effect from enzymatic catalysis (KIE_2) can be solved for algebraically. When this is done, and the errors on the measured KIEs are propagated, the enzyme KIE at pH 5 becomes 1.099 ± 0.017. This is a slight decrease from the observed effect, but is still an extremely large increase relative to the effect of 1.022 measured at pH 7. While the result at pH 5 is still only about 1/3 of the value seen for hydrolysis, lowering the pH has clearly removed a significant amount of commitment beyond the external commitment accounted for by isotope trapping experiments.

The β–dideuterium effect was also performed under high ionic strength conditions using 500 mM phosphate. This

experiment gave an observed KIE of 1.028 ± 0.018. The extremely large error associated with this measurement may be attributed to the high salt concentration causing inconsistency in the liquid scintillation counting. Nevertheless, the effect is equal to that of low salt within experimental error and was not pursued any further.

To further investigate the apparent change in rate determining steps at low pH, the primary ^{14}C isotope effect at the anomeric carbon was measured. This was done using the isotopomer pair of [^{3}H-NAc] and [2-^{14}C] CMP-NeuAc. An isotope effect of 1.019 ± 0.012 was measured at pH 5. While there is a significant amount of error associated with this experiment, the isotope effect is clearly greater than unity and close in magnitude to that of the solvolysis reaction. This suggests that the unity KIE measured on $\alpha(2\rightarrow6)$ sialyltransferase at pH 7 was actually a very small KIE that was largely suppressed by an internal commitment. If the primary ^{14}C KIE at pH 7 was unity, it should remain so even with all commitments removed.

An approach taken to investigate the possibility of reverse commitments was to try and alter the pathway for the release of products. In a random bi-bi mechanism, either of the two products (CMP and sialylLacNAc in this work) may be released first. A reverse commitment will be larger if the "stickiest" product is released last and smaller if it is released first (122). To probe this we attempted to direct that pathway for release of the sticky product, CMP, by the addition of excess CMP as well as by the addition of alkaline phosphatase to cleave CMP. By adding excess CMP, the majority of the reaction should take the pathway in which CMP is released second. When alkaline phosphatase is added to cleave CMP, the pathway for the initial release of CMP should dominate.

To address this issue, a pair of isotope effects were run under conditions where CMP would be broken down to cytidine and phosphate immediately upon exiting the active site. This was done by the addition of alkaline phosphatase to the reaction mixture. This enzyme was present in an excess which provided immediate cleavage of CMP. This was initially done with the

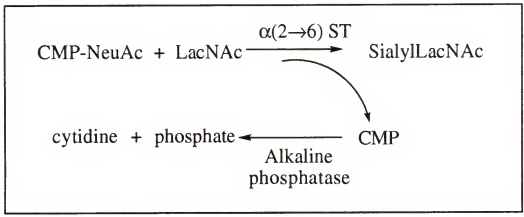


Figure 4-8. Kinetic isotope effect with alkaline phosphatase present.

isotopomer pair of [^{3}H-NAc; 3,3'-D_2] and [^{14}C-NAc] CMP-NeuAc to measure the β-dideuterium effect. An isotope effect of 1.030 ± 0.006 was observed in this experiment. The magnitude of this effect is slightly larger than without phosphatase present, but not significantly so and is within experimental error of the non-phosphatase value. The primary ^{14}C effect was also measured under these conditions using [^{3}H-NAc] and [2-^{14}C] CMP-NeuAc. An effect of 1.010 ± 0.008 was observed. This is also slightly higher than the primary ^{14}C previously measured in the absence of

phosphatase, but not significantly so taking experimental error into account.

If the slow release of CMP is diminishing the KIEs, then the addition of excess CMP should decrease the observed values substantially by increasing the reverse commitment. To address this an isotope effect was measured with an excess of CMP added to the reaction. The observed isotope effect was unchanged from those measured without CMP added. Thus, it was concluded that the off rate of CMP was not slow enough to affect the observed KIEs and that there was no significant reverse commitment.

Kinetic Isotope Effects for $\alpha(2\rightarrow6)$ Sialyltransferase with UMP-NeuAc

Another classic approach to eliminating commitments is to use an alternate substrate, which we therefore sought for $\alpha(2\rightarrow6)$ sialyltransferase. The alternate substrate should be a "slow" substrate, meaning that it is turned over by the enzyme more slowly than the natural substrate. However, the origin of the resulting slow turnover should be from a smaller value of k_{cat} not

simply weaker binding and a higher K_m. To suit our needs k_{cat} should be reduced in the rate for the isotopically sensitive step. Other characteristics of an ideal slow substrate are that it is not altered too radically such that the mode of catalysis is dramatically changed or that it undergoes alternate reactions. The candidate chosen for this work was uridine-5'-monophosphate neuraminic acid (UMP-NeuAc). The structures of UMP-NeuAc and CMP-NeuAc are shown below. The alteration made in the substrate in going from CMP-NeuAc to UMP-NeuAc is not very dramatic and is remote to the anomeric carbon of NeuAc.

The amino functionality of the cytidine ring has simply been changed to a carbonyl functionality without any changes being made proximal to the reaction center. Although the change is modest, it does remove the hydrogen bond donor at C4 of

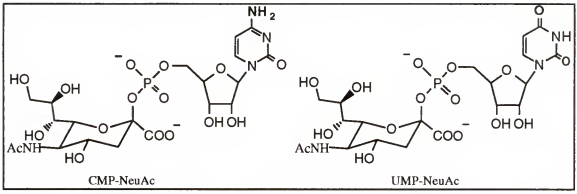


Figure 4-9. CMP-NeuAc (natural substrate) and UMP-NeuAc ("slow" substrate).

the cytidine ring and replace it with a hydrogen bond acceptor, and the hydrogen acceptor at N3 becomes a hydrogen donor. This could have a dramatic affect on the mode of binding. It is well known that hydrogen bonding plays a key role in substrate recognition and that the cytidine moiety of CMP-NeuAc is well recognized by sialyltransferases (125). As previously mentioned, though, simply reduced binding affinity will not be sufficient to eliminate internal commitments. With this in mind, the family of UMP-NeuAc radioisotopomers was synthesized to allow the isotope effects to be measured.

The first isotope effect measured was the β-dideuterium using the isotopomer pair of [^{3}H-NAc; 3,3'-D_2] and [^{14}C-NAc] UMP-NeuAc. I was delighted to find that this KIE was 1.218 ± 0.010. The isotope effect for solvolysis of this isotopomer pair was run simultaneously as a control giving a value (1.28) identical with that for solvolysis of CMP-NeuAc. While the KIE with UMP-NeuAc is not quite as large as that associated with solvolysis, it is an order of magnitude greater than the effect observed for the natural substrate CMP-NeuAc. This result suggested that most, if not all, commitments associated with the sialyltransferase catalytic cycle had been removed. It is currently unclear how this is being achieved. A reasonable explanation is simply that the substrate is bound in such a way that catalysis is hindered, thus slowing down chemistry. This could be an increase in distance of the reaction center from catalytic residues in the active site. While an argument could be made that the catalytic mechanism involving UMP-NeuAc is different than that for CMP-NeuAc, this is unlikely for a number of reasons. Firstly, the alterations made on the substrate are remote from the reaction

center so that its chemical reactivity has remained unchanged. Secondly, the same product (sialylLacNAc) is made with either substrate, which effectively does not occur in solution without enzyme present. Finally, enzymes are engineered to catalyze reactions in a specific manner, which makes it seem unlikely that there could be two modes of catalysis available.

With the promising result for the β-dideuterium KIE in hand, the next logical step was to measure the primary ^{14}C isotope effect with the UMP-NeuAc. This was accomplished using the isotopomer pair of [^{3}H-NAc] and [2-^{14}C] UMP-NeuAc. With the ^{3}H trace label now on the N-acetyl group, an effect of 1.027 ± 0.009 was measured. This is identical to that measured for solvolysis within experimental error and in the range expected for a dissociative S_N1 type mechanism. This effect is too small for any significant nucleophilic participation by the acceptor. Thus, if this transition-state is the only one leading to products (i.e., no oxocarbenium ion intermediate), the acceptor is probably bound in such a way that its hydroxyl group is just barely within bonding distance. To ensure that the [2-^{14}C] effect was indeed on

chemistry and not binding, a control was performed using the isotopomer pair of [^{3}H-NAc] and [^{14}C-NAc] UMP-NeuAc resulting in the observance of a unity KIE of 1.005 ± 0.005.

The binding isotope effect of 0.944 ± 0.010 at C9 of the polyhydroxyl tail is very interesting. A small binding effect at this position was measured with CMP-NeuAc, but this is a very large binding effect. This result suggests that this portion of the donor substrate is recognized by the enzyme and may be an important locus to consider when discussing sialyltransferase inhibitors.

Conclusions

Through an isotope partitioning experiment the external commitment to catalysis for $\alpha(2\rightarrow6)$ sialyltransferase was determined to be 1 and 1.7 at 1.6 mM and 100 mM LacNAc, respectively. This information was used to correct the observed KIEs with the natural donor substrate CMP-NeuAc. The family of KIEs measured for $\alpha(2\rightarrow6)$ sialyltransferase were much smaller than those measured for solvolysis, suggesting that there was a

significant internal commitment to catalysis associated with the reaction mechanism. The internal commitment may be a conformational change prior to glycosidic bond cleavage. The internal commitment was removed by using the alternate substrate UMP-NeuAc, which unveils the intrinsic KIEs. The intrinsic KIEs suggest that this reaction proceeds in a dissociative manner with the transition-state possessing substantial oxocarbenium ion character and little nucleophilic participation, similar to solvolysis.

Experimental

Isotope Trapping

Isotope trapping experiments were carried out in pulse-chase fashion (115). A master pulse mixture was prepared which contained 320 μM [9-^{3}H] CMP-NeuAc (121.8 mCi/mmol), 26 mU (0.079 nmols) α(2→6) sialyltransferase, 120 mM cacodylate, 4 mg/mL BSA, 0.3% Triton CF-54, pH 7. Six chase solutions were prepared with each consisting of 5 mM CDP and the appropriate concentration of LacNAc (1.6, 2, 3, 5, 16, and 100 mM). Prior to

initiating reactions, chase solutions (1 mL) and pulse solutions (30 μL) were pre-incubated at 37 °C for 2 minutes. Initiation was accomplished by adding 28 μL of the pulse directly to the chase solution while the chase was being vortexed. Vortexing was allowed to continue for 2-3 seconds before 980 μL of the mixture was applied to a column (1.5cm X 10cm) of Dowex 1X2-200 anion exchange resin (P_i form). The column was washed with 1 mM Pi buffer, pH 7, while collecting the eluent directly into liquid scintillation vials. A total of 26 fractions were collected (4 mL/fraction; 104 mL) until the entire product peak (sialylLacNAc) had eluted. The commitment factor was calculated by determining the amount of product formed relative to the amount that would have been seen if all of the enzyme bound substrate had been trapped. The experiment was run in duplicate with different batches of enzyme, as well as substrates, yielding identical results within experimental error.

Enzyme Reaction General KIE Methodology

Kinetic isotope effect (KIE) experiments used mixtures of radiolabeled isotopomeric substrates, and were therefore competitive (112). Experiments employed ca. 100,000 CPM each of the appropriate ^{3}H- and ^{14}C- labeled CMP-NeuAc isotopomers. For each experiment a master of a given $^{3}H/^{14}C$-labeled isotopomeric pair of CMP-NeuAc containing the appropriated amount of LacNAc (1.6 or 100 mM) was prepared from which aliquots were withdrawn for individual reactions and measurement of the reference $^{3}H/^{14}C$ ratio at time 0. Reaction mixtures (50-500μL, pH's 5-8) were initiated by the addition of the appropriate amount of enzyme to give 40-60% conversion in <30 minutes (68) and each was placed at 37 °C. After 40-60% conversion, unreacted substrate was isolated from the reaction mixture on a MonoQ HR10/10 column (100 mM NH_4HCO_3, 15% methanol, pH 8.0, 2 mL/min, A_{260}) and collected directly into scintillation vials (2 mL fractions). Care was taken to collect the entire CMP-NeuAc peak. The percent conversion was determined from the CMP-NeuAc and CMP peaks in the HPLC

chromatogram. The initial $^{3}H/^{14}C$ ratio was obtained in triplicate by injecting aliquots of the master mixture of labeled CMP-NeuAc on the MonoQ column and recollecting the entire CMP-NeuAc peak. The $^{3}H/^{14}C$ ratios for CMP-NeuAcs were determined by dual-channel liquid scintillation counting (channel A, 0-15 keV; channel B 15-80 keV) with each tube being counted for 10 min, and all tubes cycled through the counter 6-10 times. The internal ^{133}Ba source was used to estimate the quench parameter for each tube, which varied by ±1%. Triplicate samples of [^{14}C] CMP-NeuAc were used to determine the ratio of ^{14}C counts in channels A and B ($A:B_{14}$). Since ^{3}H is only detected in channel A, the $^{3}H/^{14}C$ ratio in a given tube was calculated with the following equation (112):

$$^{3}H/^{14}C = (\text{cpm A} - \text{cpm B} \times A:B_{14}) / (\text{cpm B} + \text{cpm B} \times A:B_{14}) \quad \text{eq. 4.7}$$

The reported value and error of a KIE represents the mean and standard deviation of three individual KIE experiments taken over

6-10 cycles through the liquid scintillation counter. The following equations were used to calculate the KIEs:

$$\beta\text{-}^{2}H\ KIE_{observed} = (^{3}H/^{14}C)_{t1/2} / (^{3}H/^{14}C)_{0} \qquad \text{eq. 4.8}$$

$$^{14}C\ KIE_{observed} = (^{14}C/^{3}H)_{t1/2} / (^{14}C/^{3}H)_{0} \qquad \text{eq. 4.9}$$

The KIEs were then corrected for %conversion using the following equation (69):

$$KIE_{corrected} = \ln(1-f) / \ln((1-f) \times KIE_{observed}) \qquad \text{eq. 4.10}$$

f = fraction of conversion

For KIEs employing stable isotopes where complete incorporation was not achieved (pro-R and ^{18}O KIEs), the following equation was used to correct the observed KIEs (111):

$$KIE_{corrected} = (KIE_{observed} - 1 + f) / f \qquad \text{eq. 4.11}$$

f = fraction of stable isotope incorporation

Variation in this methodology includes KIEs run in the presence of excess CMP or with alkaline phosphatase present in attempts to manipulate commitments. Because these changes affect the HPLC chromatogram, the % conversions could not be determined in the usual manner. In these cases, the % conversions were determined by the amount of radioactivity remaining in the unreacted product peak relative to that in the initial reaction mixture.

Isotope effects measured at pH 5 were carried out in 500 mM acetate buffer, while the effect measured at pH 8 was carried out in 250 mM Tris-HCl buffer.

Kinetic isotope effects on the $\alpha(2\rightarrow6)$ sialyltransferase system using the slow substrate UMP-NeuAc were also carried out in a similar manner. The differences were that substantially more enzyme (ca. 10 fold) was used to reach 40-60% conversion in the desired time frame (<30 minutes) and the elution time for UMP-NeuAc was slightly longer than that of CMP-NeuAc. However, this presented no difficulty in the ability to collect the entire unreacted UMP-NeuAc peak. A final difference was that

much greater concentrations (40 mM) of the acceptor substrate (LacNAc) were routinely used to achieve acceptable % conversions.

CHAPTER 5
KINETICS WITH ALPHA (2→6) SIALYLTRANSFERASE

Introduction

There are many techniques that may be utilized to probe the catalytic mechanism of an enzyme. Insight concerning the catalytic mechanism may be obtained after definition of the kinetic parameters, the kinetic mechanism, active site residues, and transition-state structure. Enzyme kinetics are often more complex than the kinetics associated with a purely chemical reaction due to the following reasons. Enzyme catalyzed reactions have substrate binding and product release steps which must be considered. The concentration of the substrate(s) can affect the step(s) in the reaction that a particular experiment probes as well. Also, the dramatic rate enhancement that enzymes often provide may be equated with fast rates for chemical steps that become as fast as binding and dissociation.

Aside from some initial kinetic characterizations, very little has been done in the way studying the mechanism of sialyltransferases. The same is true for other

glycosyltransferases with the possible exception of a fucosyltransferase. An in-depth mechanistic study has been completed on α-1,3-fucosyltransferase V which included pH-rate profiles, solvent deuterium isotope effects, and an investigation into metal requirements (34, 126). The conclusions from this work were that the mechanism involved a single catalytic base and that cleavage of the leaving group bond occurred prior to nucleophilic attack of the acceptor hydroxyl group, and the transition-state had substantial oxocarbenium ion character (34, 126). There may be similarities between this mechanism and that catalyzed by sialyltransferases, though one immediately noticeable difference is that unlike any other glycosyltransferase, sialyltransferases use a sugar nucleotide monophosphate rather than a sugar nucleotide diphosphate as the donor.

A number of kinetic experiments were performed to characterize the acid catalyzed hydrolysis of CMP-NeuAc, as well as the α(2→6) sialyltransferase reaction. These include a methanolysis and positional isotope exchange (PIX) experiment on

the solvolysis of CMP-NeuAc, determination of the kinetic parameters for $\alpha(2\rightarrow6)$ sialyltransferase with the alternate substrate UMP-NeuAc, the pH dependence of the kinetic parameters of $\alpha(2\rightarrow6)$ sialyltransferase with both CMP-NeuAc and UMP-NeuAc, and the measurement of solvent deuterium isotope effects with CMP-NeuAc and UMP-NeuAc.

Results

A stereochemical study of the solvolysis of CMP-NeuAc in aqueous methanol was conducted to give insight into the role of the carboxylate group (86). Solutions of CMP-NeuAc in 20 and 30% (v/v) methanol/water at pH 5 were allowed to hydrolyze to completion. The resulting product mixture was analyzed by ^{1}H-NMR. Integration of the unique peaks for the α-methyl and β-methyl ketals and product NeuAc showed that the α and β ketals had been formed in approximately equal proportion.

A positional isotope exchange (PIX) experiment was performed on the acid catalyzed solvolysis of CMP-NeuAc with

^{18}O located in the glycosidic bridging phosphate. ^{1}H-decoupled ^{31}P-NMR was used to analyze residual CMP-NeuAc in hydrolysis reaction mixtures. This was done to investigate the nature of the solvolysis reaction with respect to the lifetime of possible ion-pair intermediates. No measurable PIX was observed with this experiment.

The variation of the rate of catalysis with pH was also characterized using both CMP-NeuAc and UMP-NeuAc under V_{max} and V/K conditions. The pH-rate profiles with CMP-NeuAc as the donor substrate were complex (Figure 5-1). On the acid limb, both the curves showed a limiting slope of 2 and an apparent pK_a near 5.3. The V_{max} profile showed maximal velocity near pH 6, then decreased with an apparently non-integral slope towards alkaline pH. The $V/K_{CMP\text{-}NeuAc}$ profile reached a maximum limiting plateau after the acid limb pK_a of 5.3. The pH-rate profiles measured with UMP-NeuAc (Figure 5-2) were bell-shaped and fit well to equation 5.6 which describes a model requiring two ionizable groups, one in protonated form and the other in

deprotonated form. From the V_{max} plot, pK_a's of 5.5 and 9.0 were estimated for these groups, while the pK_a's were 6.2 and 8.9 from the $V/K_{UMP\text{-}NeuAc}$ plot.

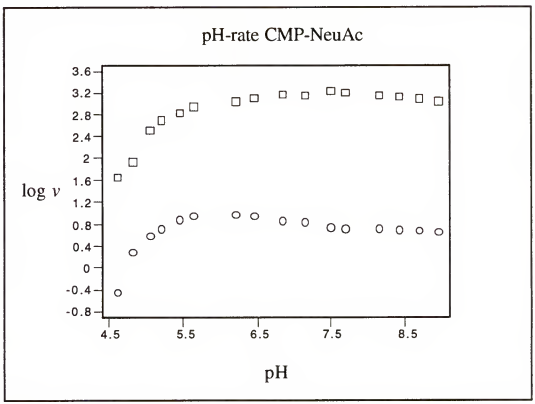


Figure 5-1. pH-rate profiles with CMP-NeuAc as donor substate. The squares represent the experimental V/K data, and the diamonds represent the V_{max} data.

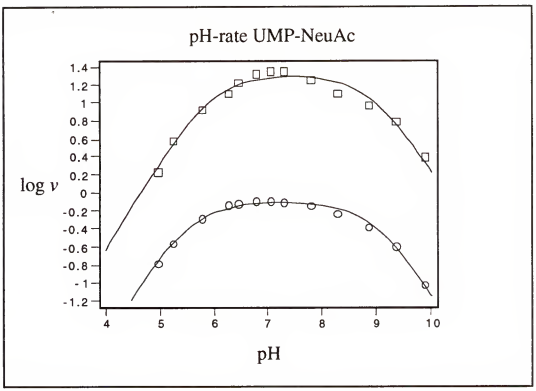
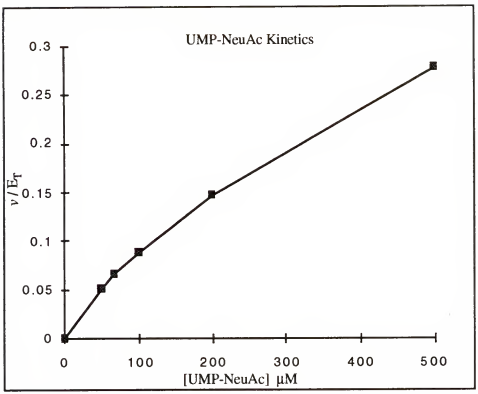


Figure 5-2. pH-rate profiles with UMP-NeuAc as donor substrate. The squares represent the experimental V/K data, the diamonds represent the V_{max} data, and the solid lines represent the fit of the data to eq. 5.3.

The kinetic parameters of $\alpha(2\rightarrow6)$ sialyltransferase with the slow substrate UMP-NeuAc were estimated by Lineweaver-Burk analysis. The data is shown in Figure 5-3 in rectangular hyperbola form and in double-reciprical form. At pH 7.5 the K_m for UMP-NeuAc was 1.2 ± 0.1 mM, the K_m for LacNAc was 4.9 ± 0.3

mM, and V_{max} was 1.8 μmol/min-mg with a corresponding k_{cat} of 1.2 s^{-1}. The results are listed Table 5-2 along with the parameters for CMP-NeuAc for comparison. The large decrease in k_{cat} as well as K_m for the alternate substrate UMP-NeuAc as compared to the natural substrate CMP-NeuAc indicate that it is useful as a slow substrate. This information is also supported by the large kinetic isotope effects measured with UMP-NeuAc.

The solvent deuterium isotope effects were measured with the altered donor substrate (UMP-NeuAc) under both V_{max} and $V/K_{UMP\text{-}NeuAc}$ conditions. Effects of 0.97 ± 0.03 and 1.12 ± 0.19 were observed for V_{max} and $V/K_{UMP\text{-}NeuAc}$, respectively. However, with CMP-NeuAc as the donor substrate, solvent isotope effects of 1.3 ± 0.1 on $V/K_{CMP\text{-}NeuAc}$ and 2.6 ± 0.3 on V_{max} were measured. A control experiment was performed in 9% (v/v) glycerol to account for the possible differences in rate due to the relative D_2O/H_2O viscosity of 1.24 (127, 128) in the glycerol reaction indicating that there is not a viscosity effect in the $\alpha(2\rightarrow6)$ sialyltransferase reaction.



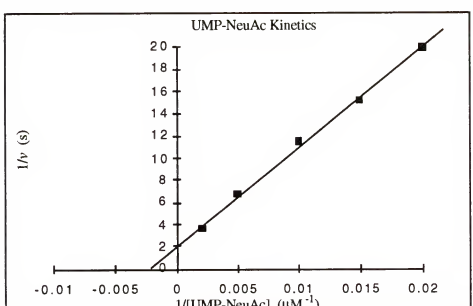


Figure 5-3. Kinetic characterization of UMP-NeuAc with $\alpha(2\rightarrow6)$ sialyltransferase.

Substrate	k_{cat} (s^{-1})	K_m (μM)
CMP-NeuAc	3	16
UMP-NeuAc	1.2	1200

Table 5-2. Kinetic parameters of CMP-NeuAc and UMP-NeuAc with rat liver α(2→6) sialyltransferase (CMP-NeuAc parameters taken from reference 47).

Discussion

Solvolysis in Aqueous Methanol

As previously discussed, the role the carboxylate group may play in catalysis is of mechanistic interest. How it is involved in the aqueous hydrolysis of CMP-NeuAc could give insight as to a possible role it may play in enzymatic catalysis. If the carboxylate is participating as a nucleophile, either through the formation of an α-lactone or by aiding in leaving group departure electrostatically, it would be expected to shield the back face of NeuAc favoring the formation of the β-anomer. If there is no

participation by the carboxylate with the leaving CMP being directly displaced by solvent in an S_N2 mechanism, only the α-anomer should be formed. If the reaction involves the formation of a sialyl oxocarbenium ion, the results could range from inversion if tight ion pairs are trapped by solvent to complete racemization if the lifetime of the cation is long enough for CMP to diffuse away. The experimental challenge in monitoring the stereochemistry of the reaction is that the hydrolysis product NeuAc undergoes rapid mutarotation (129), resulting in loss of the required stereochemical information. Methanol was included in the solvolysis reaction mixtures because it would provide configurationally stable methyl ketals as products of solvolysis, which could then be analyzed by NMR. When CMP-NeuAc was solvolysed at pH 5.0 in the presence of methanol, a 1:1 ratio of α- and β-methyl glycosides was observed. This result suggests that the mechanism proceeds through a sialyl cation which is long lived enough to allow solvent equilibration to occur, resulting in equal attack from either face. This data, along with the large β-

deuterium and small primary ^{14}C KIEs, support a solvolysis mechanism which proceeds through a sialyl cation without the involvement of nucleophilic participation of the carboxylate (86).

Positional Isotope Exchange (PIX)

As previously discussed (Chapter 2), CMP-NeuAc was synthesized with approximately 50% ^{18}O substitution at the anomeric carbon. This was done in order to perform a positional isotope exchange experiment (PIX) to probe the chemical features of the leaving group C-O bond rupture. The presence and amount of ^{18}O substitution at this position may be ascertained by ^{1}H-decoupled ^{31}P-NMR. The single phosphorous peak of CMP-NeuAc is doubled by the presence of an ^{18}O isotope shift. The experimental procedure involves measurement of the ratio of ^{18}O substitution before and after the substrate is exposed to reaction conditions

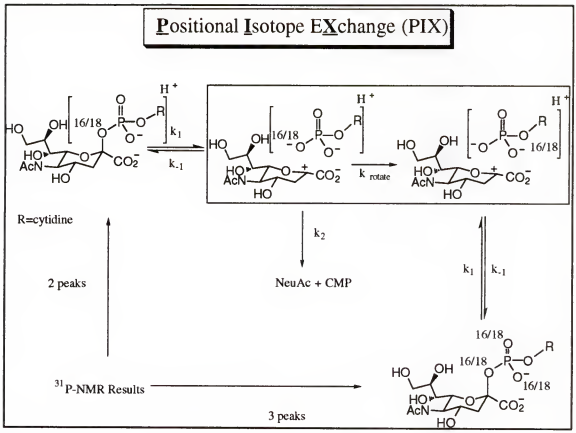


Figure 5-4. Positional isotope exchange mechanism. The site of protonation is presently unknown.

which allow cleavage of the C-O bond to occur. If the leaving group exists as a tight ion pair with the forming oxocarbenium ion, it is possible that the C-O bond could break and then reform. This will occur if k_{-1} is of similar magnitude to k_2 as shown in Figure 5-4. If this process does occur, there may be sufficient time for the phosphate group to rotate so that one of the non-

bridging oxygens becomes bridging. Since all non-bridging oxygens are initially ^{16}O, the relative size of the ^{16}O and ^{18}O phosphorous peaks should change with less ^{18}O being present and a third peak will appear from the phosphorous which now has non-bridging ^{18}O. Precedent for this experimental approach was seen in the examination of the hydrolysis of ATP in the study of T4 DNA polymerase holoenzyme (130). While the results of that study are not relevant to the present work, the experimental approach involved using ^{31}P-NMR to probe for the exchange of a bridging phosphate oxygen for a non-bridging phosphate oxygen.

Our experiment was carried out under acid catalyzed hydrolysis conditions (pH 5). The ^{1}H-decoupled ^{31}P-NMR spectrum of post-hydrolysis substrate showed no new third peak, indicating that no isotopic exchange took place. This suggests that once the sugar- phosphate bond has been cleaved, the leaving group (CMP) departs faster than the phosphate rotates and exchanges. Assuming the rotation rate at the phosphate is fast, the observed results argue that the lifetime of the tight ion pair

is short. This is also supported by the methanolysis experiment, because if the tight ion pair were long lived, a high proportion of α-methyl glycoside would have been observed which was not the case.

pH-rate Studies with CMP-NeuAc

The identity of active site residues is very useful information to have when studying the mode of enzymatic catalysis. The number of active site residues with ionizable groups can often be identified from the measurement of pH-rate profiles. In some cases, the observed pK_a's may allow preliminary identification of these groups, or they might provide subsets of likely candidates and those that are less likely. Because enzymes often utilize residues containing acidic or basic functionality, their activity usually varies with pH in the same way that simple acids and bases ionize (131). Table 5-1 is a list of normal pK_a values for these groups. As can be seen from the pK_a ranges for these groups in proteins, the pK_a of a particular

Group	pK_a Model compounds (small peptides)		Usual range in proteins
Amino acid α–CO_2H	3.6		
Asp (CO_2H)	4.0	⟸	2-5.5
Glu (CO_2H)	4.5		
His (imidazole)	6.4		5-8
Amino Acid α–NH_2	7.8		~8
Lys (ε–NH_2)	10.4		~10
Arg (guanidine)	~12		---
Tyr (OH)	9.7		9-12
Cys (SH)	9.1		8-11
Phosphates	1.3, 6.5		---

Table 5-1. pK_a's of ionizing groups (Taken from reference 131).

residue is sensitive to its surroundings and is not easily predicted.

However, the data may be used in conjunction with other experiments to give a reasonable idea of the identity of catalytic residues. The slope of a plot of log (rate) vs pH will also give the number of ionizing groups involved in catalysis (131) .

The pH-rate profiles measured using CMP-NeuAc as the donor substrate and LacNAc on the $\alpha(2\rightarrow6)$ sialyltransferase system are shown in Figure 5.2. These curves demonstrate the kinetic complexity of the $\alpha(2\rightarrow6)$ sialyltransferase catalytic cycle. While the acid limb of both profiles suggest that a base isnecessary for catalysis, the plateaus seen in the alkaline region are unusual. The solution hydrolysis is specific-acid catalyzed (86), and it seems reasonable to expect that sialyltransferase would facilitate loss of CMP by providing a proton. This is a common catalytic strategy for glycosylases (132). The plateau portions of the profiles may be misleading because the kinetics are steady-state. In such cases, shifted pK_a's are common, and therefore an ionization of an acid group might be shifted outside the experimental range. The function of a catalytic general base would possibly involve deprotonation of the incoming acceptor hydroxyl group nucleophile which is ultimately required for completion of the reaction. On the other hand, the ionization on the acid limb may not represent a "base" in the Bronsted sense at all. It could represent a group that is required for catalysis, and

must be deprotonated. For example, such a group could provide a critical hydrogen bond to properly orient substrate for catalysis or stabilize an oxocarbenium ion like transition-state. To better define the pH-rate relationships, we have measured the pH-rate profiles with the "slow" substrate UMP-NeuAc.

pH-rate UMP-NeuAc

The pH-rate profiles for UMP-NeuAc were measured to explore its altered kinetics. This was done in the pH range of 5-10 under both V_{max} and $V/K_{UMP\text{-}NeuAc}$ conditions. The resulting curves are shown in Figure 5.3. The pH-rate profiles for both cases (V_{max} and $V/K_{UMP\text{-}NeuAc}$) were well-fit to bell-shaped curves described by eq. 5.1. In this equation C is the maximal value of

$$\log v = \log \{C/1+([H^+]/K_{a1})+(K_{a2}/[H^+])\} \qquad \text{eq. 5.1}$$

V_{max} or V/K if all the groups are in the correct protonation state, and K_{a1} and K_{a2} are the acid dissociation constants of the catalytically required groups on the acidic and basic limbs,

respectively. This result demonstrates how the altered substrate has greatly simplified the kinetic behavior of the enzyme. The pK_a's identified in these experiments are consistent with sialyltransferase requiring two ionizable groups for catalysis. Since UMP-NeuAc does not have any ionizable groups with pK_a's in this region, the ionizations identified in the pH-rate profiles are attributed to free enzyme and the enzyme substrate Michaelis complex for V/K and V_{max}, respectively (133).

It is likely that the function of the protonated group is to aid in the cleavage of the sugar-phosphate bond, while the ionized group may function as a general base which interacts with the nucleophilic hydroxyl group of the acceptor substrate, or stabilizes the oxocarbenium ion transition-state.

Kinetic Characterization of UMP-NeuAc with $\alpha(2\rightarrow6)$ Sialyltransferase: Lineweaver-Burk Analysis

The ideal "slow" substrate candidate would not necessarily be bound by the enzyme less tightly (K_m effect), but would have slower chemical rate of reaction (V_{max} effect) (134). This is

necessary in order to eliminate or minimize any internal commitments in the mechanism. Therefore, it was desirable to measure the kinetic parameters of UMP-NeuAc with $\alpha(2\rightarrow6)$ sialyltransferase. This was accomplished by measuring the reaction velocity at varied substrate concentrations and fitting the kinetic data to eq. 5.2.

$$v = [E]_0[S]k_{cat} / K_m + [S] \qquad \text{eq. 5.2}$$

$$1/v = (1/V_{max}) + (K_m/V_{max})[S] \qquad \text{eq. 5.3}$$

The data is first plotted in the rectangular hyperbola form of eq. 5.2 as presented in Figure 5.3. The data may then be plotted in the double-reciprical form of the Lineweaver-Burk equation (eq. 5.3) (Figure 5-3) to give the desired information. The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation using the computer program MacCurveFit and are listed in Table 5-2 along with the parameters for the natural substrate CMP-NeuAc for comparison. The important comparison to be made for this work is the decrease (>2 fold) in k_{cat} for UMP-

NeuAc relative to CMP-NeuAc. The K_m for UMP-NeuAc is 75 times higher than that of CMP-NeuAc indicating that it is much less tightly bound by sialyltransferase. This gives about a 150 fold reduction in k_{cat}/K_m relative to that of CMP-NeuAc, which supports the idea that UMP-NeuAc is an ideal slow substrate for sialyltransferase.

Solvent Deuterium Isotope Effects

The solvent isotope effects measured for $\alpha(2\rightarrow6)$ sialyltransferase were quite interesting. When the alternate substrate UMP-NeuAc was used as the donor, unity effects were observed under both V_{max} and $V/K_{UMP\text{-}NeuAc}$ conditions. However, with CMP-NeuAc as the donor substrate the observed solvent deuterium effects were 2.6 ± 0.3 on V_{max} and 1.3 ± 0.1 on $V/K_{UMP\text{-}NeuAc}$. This suggests that a step involving solvent exchangeable protons is partially rate limiting and distinct from cleavage of the glycosidic bond. The disappearance of the solvent effect when UMP-NeuAc is used is attributed to glycosidic bond cleavage becoming the dominant rate-determining step.

The doubling of the isotope effect from 1.3 to 2.6 may indicate a removal of commitment. The isotope effect on V_{max} will be insensitive to the off-rate for substrate, whereas the solvent effect on $V/K_{CMP\text{-}NeuAc}$ would include this factor. As discussed earlier an external commitment does exist for CMP-NeuAc.

Chemically speaking, a solvent isotope effect of 2.6 is sufficiently large to be indicative of a proton in flight, or a catalytic solvation bridge as described by Schowen (135). Because proton transfer between heteroatoms are considered to usually be fast, the observed isotope effect might not be rate-limiting in itself, but rather it might be part of a step which is rate-limiting. For example, a conformational change which involved changes in bending to solvent exchangeable hydrogens could be operative. Further study will be required to address this issue more fully.

Control experiments were run to ensure that the solvent isotope effects did not originate from differences in the viscosities of H_2O and D_2O. This could occur if there is a step in

the kinetic mechanism which is affected by the viscosity of the reaction mixture. There was no difference in the rates with or without glycerol indicating the observed solvent deuterium effects were not associated with solvent viscosity.

Conclusions

The work in this chapter has further characterized both the solvolysis and enzymatic transfer of CMP-NeuAc. The methanolysis work indicated that in solvolysis the oxocarbenium ion was long lived enough to be trapped equally from faces without nucleophilic participation of the carboxylate group. The absence of any detectable PIX with CMP-NeuAc indicates that the phosphate group of the departing CMP moiety is not able to rotate and re-form the bond with the NeuAc oxocarbenium ion before it leaves. This indicates that the life of the tight ion pair is short, which is also supported by the observation in the methanolysis experiment that the leaving group CMP does not block the β face of NeuAc from nucleophilic attack.

By determination of the kinetic parameters for the altered substrate UMP-NeuAc, it was demonstrated that it was a "slow" substrate for sialyltransferase which is consistent with the KIE results.

The pH-rate profiles measured with CMP-NeuAc were complex, but showed the presence of a protonated group necessary for catalysis. Upon switching to UMP-NeuAc as the donor substrate, the pH-rate curves became bell-shaped and showed that both a protonated and an unprotonated group were necessary for catalysis. The differences in the curves for the two substrates is attributed to a change in the rate-determining step when UMP-NeuAc is the donor substrate.

No significant solvent deuterium isotope effects were seen with UMP-NeuAc, however, large effects were observed with CMP-NeuAc. This can be explained by the step associated with the solvent effect no longer being rate-determining when UMP-NeuAc is used, thus masking the solvent effects. We postulate that this is a step occurring prior to glycosidic bond cleavage, and is possibly a conformational change.

Experimental

Solvolysis of CMP-NeuAc in Aqueous Methanol

Reactions (1.0 mL) were conducted in 1.5 mL polypropylene microcentrifuge tubes and consisted of 6 mM CMP NeuAc, 200 mM CD_3COOH, pH 5.0 or 6.0 and 20% or 30% (v/v) methanol. The reactions were maintained at 37 °C and monitored by HPLC until CMP-NeuAc consumption was greater than 98% (14 h, pH 5.0; 72 h, pH 6.0). Reaction mixtures were concentrated *in vacuo* and exchanged against D_2O four times to remove traces of methanol. ^{1}H-NMR analysis was used to determine the ratio of α- and β-methyl ketosides (136) of NeuAc, 2,3-dehydro-NeuAc, and NeuAc based on integration of resonances at 5.70 (CH, vinylic 2,3-dehydro-NeuAc), 3.32 (OCH_3, α-methyl ketoside), 3.18 (OCH_3, β-methyl ketoside), and 2.19 ($H\text{-}3_{eq}$, β -NeuAc anomer). The total NeuAc concentration was calculated by multiplying the observed integral for the β-NeuAc anomer by 1.087 based on the reported α / β ratio of 92:8 (which we also confirmed) (137). The α- and β-

methyl glycosides' identities were confirmed by addition of authentic samples of the glycosides to separate aliquots of solvolysis reaction mixtures. Controls established that the product methyl glycosides were stable to the reaction conditions.

Dowex Column Methodology (53)

The product analysis of the kinetic experiments was carried out on Dowex mini-columns. Aliquots (20-60 μL) were withdrawn at the appropriate times and quenched into 500 μL ice cold 5 mM phosphate buffer. Each mixture was then vortexed and 500 μL of this was then applied to a column (0.5 cm X 4 cm) of Dowex 1X2-200 anion exchange resin (P_i form). The resin was then washed with an additional 3.5 mL of 5 mM P_i buffer to elute product. The eluent was collected directly into a liquid scintillation vial for counting.

Vmax pH-rate profile (saturating CMP-NeuAc and LacNAc)

The pH behavior of V_{max} for sialyltransferase was estimated by holding [CMP-NeuAc] and [LacNAc] at 10^*K_m values.

The reactions were carried out in either 800mM Tris/Bis Tris (pH 6-9) or 800 mM sodium acetate buffer (pH 4-6) at 37 °C. The buffers were adjusted to the appropriate pH at 37 °C (pH's used were 4.61, 4.82, 5.06, 5.20, 5.45, 5.63, 6.20, 6.45, 6.85, 7.15, 7.49, 7.70, 8.14, 8.41, 8.69, 8.93). A stock solution containing 700 μM [1-^{14}C] CMP-NeuAc (60,000 CPM/reaction; 1.3 mCi/mmol) was prepared as well as 70 μL stocks of each buffer at the appropriate pH containing 40 mM LacNAc. All reactions were carried out at 37 °C and were initiated by adding 30 μL of the 40 mM LacNAc/buffer stock to a mixture containing 30 μL of the [1-^{14}C] CMP-NeuAc stock and 5 μL of a 0.09 mU/μL sialyltransferase stock. All solutions were pre-incubated at 37 °C for 1 minute prior to initiating the reaction. Aliquots (20 μL) were withdrawn at 2, 4, and 6 minutes and the formation of product was determined with the Dowex column methodology. Control reactions were done for all pH's below 6 to correct rates for background hydrolysis (background hydrolysis is insignificant above pH 6 during the time course of the assay). These were

completed in an identical manner with 5 μL of dH_2O being substituted for enzyme.

V/K pH-Rate (saturating [CMP-NeuAc] and low [LacNAc])

The pH behavior of V/K_{LacNAc} was estimated by holding [CMP-NeuAc] at 10^*K_m with [LacNAc] at 370 μM, which is about 1/5 its K_m value (53). The reactions were carried out in either 800 mM Tris/Bis Tris (pH 6-9) or 800 mM sodium acetate buffer (pH 4-6) at 37 °C. The buffers were adjusted to the appropriate pH at 37 °C (pH's used were 4.61, 4.82, 5.06, 5.20, 5.45, 5.63, 6.20, 6.45, 6.85, 7.15, 7.49, 7.70, 8.14, 8.41, 8.69, 8.93). A stock solution containing 700 μM [1-^{14}C] CMP-NeuAc (60,000 CPM/reaction; 1.3 mCi/mmol) was prepared as well as 70 μL stocks of each buffer at the appropriate pH containing 800 μM LacNAc. All reactions were carried out at 37 °C and were initiated by adding 30 μL of the 800 μM LacNAc/buffer stock to a mixture containing 30 μL of the [1-^{14}C] CMP-NeuAc stock and 5 μL of a 0.3 mU/μL sialyltransferase stock. All solutions were pre-incubated at 37 °C for 1 minute prior to use. Aliquots (20

μL) were withdrawn at 2, 4, and 6 minutes and the formation of product was determined with the Dowex column methodology. Control reactions were done for all pH's below 6 to correct rates for background hydrolysis (background hydrolysis is insignificant above pH 6 during the time course of the assay). These were completed in an identical manner with 5 μL of dH_2O being substituted for enzyme.

V/K pH-Rate (saturating [LacNAc] and low [CMP-NeuAc])

The pH behavior of $V/K_{CMP\text{-}NeuAc}$ was estimated by holding [LacNAc] at $10*K_m$ with [CMP-NeuAc] at 44 nM. The reactions were carried out in either 800 mM Tris/Bis Tris (pH 6-9) or 800 mM sodium acetate buffer (pH 4-6) at 37 °C. The buffers were adjusted to the appropriate pH at 37 °C (pH's used were 4.61, 4.82, 5.06, 5.20, 5.45, 5.63, 6.20, 6.45, 6.85, 7.15, 7.49, 7.70, 8.14, 8.41, 8.69, 8.93). A stock solution containing [9-^{3}H] CMP-NeuAc (60,000 CPM; 10 mCi/mol) was prepared as well as 75 μL stocks of each buffer at the appropriate pH containing 40 mM LacNAc. All reactions were carried out at 37 °C and were

initiated by adding 30 μL of the 40 mM LacNAc/buffer stock to a mixture containing 30 μL of the [9-^{3}H] CMP-NeuAc stock and 5 μL of a 0.1 mU/μL sialyltransferase stock. All solutions were pre-incubated at 37 °C for 1 minute prior to use. Aliquots (20 μL) were withdrawn at 2, 4, and 6 minutes and the formation of product was determined with the Dowex column methodology. Control reactions were done for all pH's below 6 to correct rates for background hydrolysis (background hydrolysis is insignificant above pH 6 during the time course of the assay). Control reactions were completed in an identical manner with 5 μL of dH_2O being substituted for enzyme.

pH-rate controls

Control experiments were performed to ensure saturation of the enzyme by UMP-NeuAc or LacNAc at the pH extremes (pH 5 and pH 10), as well as to check for any irreversible inactivation of the enzyme at low pH. The saturation checks were performed by running two side by side reactions in the same manner as the pH-rate kinetics. One reaction contained the concentrations used in

the corresponding pH-rate experiment, while the second contained an increased concentration of the substrate in question. The rates of the two reactions were then compared. To check for saturation of UMP-NeuAc, reactions were run at 2 mM and 3 mM [UMP-NeuAc] while keeping LacNAc at 40 mM. At pH 10 the rates were identical within experimental error, and at pH 5 the 2 mM UMP-NeuAc reaction was at $0.85*V_{max}$ relative to the 3 mM UMP-NeuAc reaction. To check for saturation of LacNAc, reactions were run at 40 mM and 60 mM [LacNAc] while keeping UMP-NeuAc at 2 mM. At pH 10 the rates were identical within experimental error, and at pH 5 the 40 mM LacNAc reaction was at $0.81*V_{max}$ relative to the 60 mM LacNAc reaction.

The enzyme stability at pH 4.2 and 10.0 was investigated by incubating 3 mU of sialyltransferase in 55 μL 800 mM acetate buffer (pH 4.2) or (pH 10.0) at 37 °C. Aliquots (15 μL) were removed at 2,4 and 6 minutes and added to 15 μL of reaction mixture containing saturating [9-^{3}H] CMP-NeuAc and LacNAc. These reactions were allowed to proceed for 5 minutes at 37 °C before being quenched with cold 5 mM P_i buffer and applied to

Dowex columns for product quantification. An identical reaction was run without pre-incubating the enzyme at the pH extremes as well as a control reaction that did not contain enzyme to account for background hydrolysis. The enzyme activity did not change within experimental error with incubation at low or high pH indicating that the enzyme was stable under these conditions.

Lineweaver-Burk for UMP-NeuAc with $\alpha(2\rightarrow6)$ sialyltransferase

Five reaction mixtures (67 μL each) were prepared with the following UMP-NeuAc concentrations: 500, 200, 100, 67, and 50 μM. Each reaction mixture was made 20 mM LacNAc, 40 mM cacodylate, 0.2 mg/ml BSA, 0.2% Triton CF-54, pH 7.5. Each reaction contained 0.044 μCi (64,000 CPM) of [9-^{3}H] UMP-NeuAc. The mixtures were pre-incubated at 37 °C for 1 minute. Reactions were initiated by the addition of 4.3 mU enzyme (8 U/mg). Aliquots (20 μL) were removed at 5, 10, and 15 minutes and the formation of product was determined with the Dowex column methodology. Kinetic parameters were determined by Lineweaver-Burk analysis.

Five reaction mixtures (67 μL each) were prepared with the following LacNAc concentrations: 20, 4, 2, 1.33, and 1 mM. Each reaction mixture was made 2 mM UMP-NeuAc, 40 mM cacodylate, 0.2 mg/ml BSA, 0.2% Triton CF-54, pH 7.5. Each reaction contained 0.044 μCi (64,000 CPM) of [9-^{3}H] UMP-NeuAc. The mixtures were pre-incubated at 37 °C for 1 minute. Reactions were initiated with the following amounts of enzyme ([LacNAc] is listed in parenthesis): 5.7 mU (20 mM), 7.1 mU (4 mM), 8.6 mU (2 mM), 12.9 mU (1.33 and 1 mM). Aliquots (20 μL) were removed at 10 and 20 minutes and the formation of product was determined with the Dowex column methodology. Kinetic parameters were determined by Lineweaver-Burk analysis.

pH-rate for UMP-NeuAc with α(2→6) sialyltransferase

All pH-rate experiments with UMP-NeuAc were run in buffer consisting of 250 mM of the following: sodium acetate, cacodylate, Bis Tris propane, and CHES. Each also contained 0.2 mg/mL BSA and 0.2 % Triton CF-54. The pH of 3 mL aliquots of this buffer were individually adjusted with either conc HCl or 5 N

NaOH at 37°C. Reactions were run in the pH range 5-10 with control reactions being run at the pH extremes to account for any background hydrolysis of substrate.

For reactions run under V_{max} conditions a stock was prepared in each buffer containing 40 mM LacNAc. A stock of [9-^{3}H] UMP-NeuAc (4 mM; 120,000 CPM/reaction; 0.69 μCi/μmol) was prepared to be used at each pH. Enzyme was then added (1 mU for pH 6-8.5; 1.2 mU for pH 5-6 and 8.5-10) to each buffer/LacNAc mixture and incubated at 37 °C for 1 minute. Reactions were initiated by adding an equal volume of the UMP-NeuAc stock final volume = 60 μL). Control reactions were identical to enzyme reactions with the exception of water being added instead of enzyme. Aliquots (20 μL) were removed at 8, 16, 24 minutes and the formation of product was determined with the Dowex column methodology.

For reactions run under V/K conditions a stock was prepared in each buffer containing 40 mM LacNAc. A stock of UMP-NeuAc (300 nM; 10 Ci/mmol) was prepared to be used at each pH. Enzyme was added (1 mU for pH 6-8.5; 1.2 mU for pH 5-6 and 8.5-

10) to each buffer/LacNAc mixture and incubated at 37 °C for 1 minute. Reactions were initiated by adding an equal volume of the UMP-NeuAc stock final volume = 60 μL). Control reactions were identical to enzyme reactions with the exception of water being added instead of enzyme. Aliquots (20 μL) were removed at 8, 16, 24 minutes and the formation of product was determined with the Dowex column methodology.

Solvent Deuterium Kinetic Isotope Effects with CMP-NeuAc

For effects under V_{max} conditions, a 1 mL solution was prepared consisting of 500 μM [1-^{14}C] CMP-NeuAc (1.44 mmols/mCi), 16mM LacNAc, 40 mM cacodylate, 0.2 mg/mL BSA, and 0.2% Triton CF-54 (pH 6.5). Half of this mixture was rotovapped to dryness *in vacuo* and exchanged with $^{2}H_2O$ (99.9% ^{2}H; Cambridge Isotope Laboratories, Inc.) three times and brought to a final volume of 500 μL in $^{2}H_2O$. It was determined that this solution had a pD of 6.6 by addition of 0.4 to the pH meter reading (pD = pH meter reading + 0.4) due to the isotope effect on the pH probe (138). Reactions (220 μL) were run in duplicate in H_2O and

^2H_2O. Reactions were initiated by the addition of 0.38 mU $\alpha(2\rightarrow6)$ sialyltransferase and were run at 37 °C, with 65 μL aliquots removed at 8, 16, 24 minutes and the formation of product was determined with the Dowex column methodology.

For effects under V/K (CMP-NeuAc) conditions, a 1 mL solution was prepared consisting of 11 nM [1-^{14}C] CMP-NeuAc (30 mmols/mCi), 16mM LacNAc, 40 mM cacodylate, 0.2 mg/mL BSA, and 0.2% Triton CF-54 (pH 6.5). Half of this mixture was rotovapped to dryness *in vacuo* and exchanged with ^2H_2O (99.9% ^2H; Cambridge Isotope Laboratories, Inc.) three times and brought to a final volume of 500 μL in ^2H_2O. It was determined that this solution had a pD of 6.6 by addition of 0.4 to the pH meter reading (pD = pH meter reading + 0.4) due to the isotope effect on the pH probe (138). Reactions (220 μL) were run in duplicate in H_2O and ^2H_2O. Reactions were initiated by the addition of 0.25 mU $\alpha(2\rightarrow6)$ sialyltransferase and were run at 37°C, with 65 μL aliquots removed at 8, 16, 24 minutes and the formation of product was determined with the Dowex column methodology.

A control experiment was performed to address the possibility that the solvent effects could be due to the viscosity of D_2O being 1.24 that of H_2O at 37 °C (2). A solution (200 μL) was prepared consisting of [1-^{14}C] CMP-NeuAc (30 mCi/mmol), 16 mM LacNAc, 40 mM cacodylate, 0.2 mg/ml BSA, 0.2% Triton CF-54, pH 6.5. This was divided into two 91 μL reactions with 9 μL H_2O added to one and 9 μL glycerol added to the other to give a viscosity of 1.24 (127). The glycerol solution has a viscosity 1.24 that of the H_2O solution (128). Reactions were initiated by the addition of 0.19 mU of α(2→6) sialyltransferase, with 30 μL aliquots being removed at 8, 16, 24 minutes and the formation of product was determined with the Dowex column methodology.

Solvent Isotope Effects with UMP-NeuAc

For measurement of the solvent deuterium isotope effect under V_{max} conditions, a stock solution containing 4 mM [9-^{3}H] UMP-NeuAc (40,000 CPM/reaction; 0.69 μCi/μmol) and 40 mM LacNAc was prepared in 250 mM Bis-Tris propane buffer, 0.2 mg/mL BSA, 0.2% Triton CF-54, pH 7.8. Half of the reaction

mixture was retained for the reaction in H_2O while the other half was rotovapped to dryness *in vacuo* and brought up in 99.9 % D_2O (Cambridge Laboratories, Inc.) three times. It was determined that this solution had a pD of 7.6 by addition of 0.4 to the pH meter reading (pD = pH meter reading + 0.4) due to the isotope effect on the pH probe (138). Reactions were incubated at 37 °C for 1 minute before being initiated by the addition of 5 μL of a 1.2 mU/5 μL enzyme stock solution of α(2→6) sialyltransferase (final volume = 65 μL). Aliquots (20 μL) were removed at 6, 12, 18 minutes and the formation of product was determined with the Dowex column methodology.

For measurement of the solvent deuterium isotope effect under V/K conditions, a stock solution containing 0.3 μM [9-^{3}H] UMP-NeuAc (10 Ci/mmol) and 40 mM LacNAc was prepared in 250 mM Bis-Tris propane buffer, 0.2 mg/mL BSA, 0.2% Triton CF-54, pH 7.8. Half of the reaction mixture was retained for the reaction in H_2O while the other half was rotovapped to dryness *in vacuo* and brought up in 99.9 % D_2O (Cambridge Laboratories, Inc.) three times. It was determined that this solution had a pD of 7.6 by

addition of 0.4 to the pH meter reading (pD = pH meter reading + 0.4) due to the isotope effect on the pH probe (1139). Reactions were incubated at 37 °C for 1 minute before being initiated by the addition of 5 μL of a 1.2 mU/5 μL enzyme stock solution of α(2→6) sialyltransferase (final volume = 65 μL). Aliquots (20 μL) were removed at 6, 12, 18 minutes and the formation of product was determined with the Dowex column methodology.

Positional Isotope Exchange on acid catalyzed hydrolysis of CMP-NeuAc

[2-^{18}O]CMP-NeuAc (15 mg, 0.024 mmol) was brought up in 750 μL D_2O, pH 5 and placed at 37 °C for 1 hour. An analytical HPLC injection indicated 10% hydrolysis. The sample was then HPLC purified on a MonoQ anion exchange column, desalted with Amberlite IR-120H$^+$ cation exchange resin, and rotovapped to dryness *in vacuo*. The sample was then brought up in 750 μL D_2O with 0.05 equivalents EDTA added. The exchange of bridging and

nonbridging ^{18}O was then probed by proton decoupled ^{31}P-NMR at 121.4 MHz on the Gemini-300.

CHAPTER 6
KINETIC INVESTIGATION OF ALPHA (2→3) SIALYLTRANSFERASE

Introduction

The reaction products of the different sialyltransferases are involved in different biological processes; therefore, it would be of great interest if the work performed on α(2→6) sialyltransferase could be extended to other members of the family to assist in inhibitor design. Inhibitors that are effective against multiple sialyltransferases could be useful, but as drugs they might have side effects associated with them resulting from a cross-reactivity with other members of the sialyltransferase family. Thus, it is imaginable that inhibitors specific for a particular sialyltransferase would be valuable. Studies have shown that the α(2→6) and α(2→3) enzymes are inhibited similarly with respect to substrate analogs, but this may not be the case for transition-state mimics (55).

An α(2→3) sialyltransferase catalyzes the reaction shown below in Figure 6-1. The difference between this reaction and that of α(2→6) sialyltransferase is that the glycosidic linkage

involves the C3 hydroxyl group of the acceptor sugar rather than the C6 hydroxyl group. Towards the goal of characterizing other members of the sialyltransferase family, selected isotope effects and kinetic experiments have been performed on rat liver α(2→3) sialyltransferase, which was obtained in recombinant form from Calbiochem.

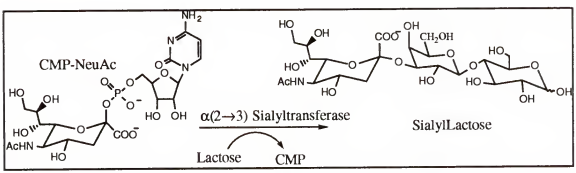


Figure 6-1. α(2→3) sialyltransferase catalyzed reaction.

Results

The kinetic isotope effects measured for α(2→3) sialyltransferase are listed in Table 6-1. A β-dideuterium effect of 1.027 ± 0.003 and a primary ^{14}C effect of 1.011 ± 0.009 were

Isotopomer Pair	Type of KIE	Value
[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc (lactose as acceptor)	β-secondary	1.027 ± 0.003
[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] CMP-NeuAc (galactose as acceptor)	β-secondary	1.170 ± 0.010
[1-^{3}H-N-acetyl; 3,3'-$^{2}H_2$]; [1-^{14}C-N-acetyl] UMP-NeuAc (lactose as acceptor)	β-secondary	1.160 ± 0.010
[1-^{3}H-N-acetyl]; [2-^{14}] CMP-NeuAc (lactose as acceptor)	primary ^{14}C	1.011 ± 0.009
[1-^{3}H-N-acetyl]; [2-^{14}] UMP-NeuAc (lactose as acceptor)	primary ^{14}C	1.027 ± 0.010

Table 6-1. KIEs measured for α(2→3) sialyltransferase. All KIEs measured at pH 7.

seen using the indicated isotopomeric pairs of CMP-NeuAc with lactose as the acceptor. The pattern of KIEs is similar to that seen for α(2→6) sialyltransferase, but the lower β-secondary effects are significantly lower suggesting an earlier transition-state for the α(2→3) sialyltransferase.

To remove commitments, effects were measured with a slow donor substrate (UMP-NeuAc) or a slow acceptor substrate (galactose). With the slow donor substrate (UMP-NeuAc) a β-dideuterium effect of 1.160 ± 0.012 and a primary ^{14}C effect of

1.027 ± 0.010 were measured. Using the slow acceptor substrate galactose, a β-dideuterium effect of 1.170 ± 0.01 was measured. The kinetic parameters for UMP-NeuAc and lactose were measured for α(2→3) sialyltransferase. With varied [UMP-NeuAc] and saturating lactose, values of 0.29 s^{-1} and 300 ± 7 μM were determined for k_{cat} and K_m(UMP-NeuAc), respectively. With varied [lactose] and saturating [UMP-NeuAc], values of 0.25 s^{-1} and 95 ± 12 mM were determined for k_{cat} and K_m(lactose), respectively.

Substrate	k_{cat} (s^{-1})	K_m (μM)
CMP-NeuAc	16.7	45
UMP-NeuAc	0.29	300
Galactose (acceptor)	1.7	——

Table 6-2. Kinetic parameters of α(2→3) sialyltransferase. (Parameters for CMP-NeuAc and Galactose taken from reference 53).

The kinetic parameters of UMP-NeuAc and lactose are listed in Table 6-2 along with the CMP-NeuAc parameters for comparison.

Solvent isotope effects of 0.93 ± 0.2 and 2.2 ± 0.3 were measured with CMP-NeuAc under V_{max} and $V/K_{CMP\text{-}NeuAc}$ conditions, respectively. Solvent effects of 1.2 ± 0.2 and 1.9 ± 0.1 were measured with UMP-NeuAc under V_{max} and $V/K_{UMP\text{-}NeuAc}$ conditions.

Discussion

Kinetic Parameters for UMP-NeuAc with α(2→3) Sialyltransferase

The kinetic parameters with the altered nucleotide substrate UMP-NeuAc were estimated by fitting the kinetic data to the Michaelis-Menten equation using MacCurveFit in order to assess its utility as a slow donor substrate. The measured parameters are listed below in Table 6-2. The substantial decrease in k_{cat} (60 fold) suggested that UMP-NeuAc would be an ideal substrate to measure intrinsic KIEs on the α(2→3) sialyltransferase system, as it was for α(2→6) sialyltransferase.

The K_m for this substrate was also significantly increased relative to that of the natural donor substrate CMP-NeuAc.

Kinetic Isotope Effects Using the Natural Substrates (CMP-NeuAc and Lactose)

The first isotope effect measured on the $\alpha(2\rightarrow3)$ sialyltransferase system was the β-dideuterium KIE using the two natural substrates, CMP-NeuAc and lactose. This was done in the same manner as for $\alpha(2\rightarrow6)$ sialyltransferase. Using the isotopomer pair of [^{3}H-NAc;3,3'-D_2] and [^{14}C-NAc] CMP-NeuAc, an isotope effect of 1.027 ± 0.003 was measured. This is identical to that measured for $\alpha(2\rightarrow6)$ sialyltransferase within experimental error. This was an initial indication that the two systems have similar transition-states. This result suggests that there is little charge build-up at the anomeric carbon in the transition-state. However, with the likely possibility of commitments, no firm conclusions can be drawn from this result alone.

To further explore the $\alpha(2\rightarrow3)$ mechanism, the primary ^{14}C isotope effect was measured using the isotopomeric pair of [^{3}H-NAc] and [2-^{14}C] CMP-NeuAc and lactose as the acceptor substrate. A primary ^{14}C isotope effect of 1.011 ± 0.009 was observed as compared to the unity KIE measured on the $\alpha(2\rightarrow6)$ enzyme. There was a large error associated with this effect which makes it difficult to interpret, but clearly shows that there is only a small primary ^{14}C effect at this position. This value is smaller than expected for a dissociative type mechanism (1.02-1.05) and very much smaller than expected for an associative type mechanism (1.08-1.12) (101). Again, this is similar to the result observed for the $\alpha(2\rightarrow6)$ system.

Kinetic Isotope Effects Using Unnatural Substrates (UMP-NeuAc and Galactose)

To address commitment issues, isotope effects were measured using alternate "slow" substrates. While this is the same general approach implemented to remove commitments on

the $\alpha(2\rightarrow6)$ system, a difference is that an alternate acceptor substrate was initially used for the $\alpha(2\rightarrow3)$ system. A brief review of the literature revealed galactose to be an ideal candidate for this work (139). The K_m for galactose for $\alpha(2\rightarrow3)$ sialyltransferase is 268 ± 90 mM and the relative V_{max} is ≈0.1 that of lactose. With a V_{max} diminished to this extent, any commitments associated with the catalytic cycle should be eliminated. Using the isotopomeric pair of [^{3}H-NAc;3,3'-D_2] and [^{14}C-NAc] CMP-NeuAc in the presence of 500 mM galactose, an isotope effect of 1.170 ± 0.010 was measured. The magnitude of this effect indicates that much or all of the commitment has been eliminated, however, this effect is not quite as large as the value observed for $\alpha(2\rightarrow6)$ sialyltransferase using UMP-NeuAc (1.22). The KIE was repeated using the isotopomeric pair of [^{3}H-NAc;3,3'-D_2] and [^{14}C-NAc] UMP-NeuAc and lactose as the acceptor to determine if the intrinsic isotope effects are different for these two enzymes, or if galactose was simply not slow enough of a substrate to eliminate all commitments. This experiment

resulted in an observed isotope effect of 1.160 ± .012 which is identical within experimental error to the value seen for the slow acceptor substrate galactose. Because V_{max} has been diminished 60 fold with UMP-NeuAc and 10 fold with galactose and these two slow substrates give identical observed isotope effects, this strongly suggests intrinsic effects are being measured. Interestingly, the effects are significantly lower than those observed for $\alpha(2\rightarrow6)$ sialyltransferase. Either there is less positive charge at the reaction center during the $\alpha(2\rightarrow3)$ transition-state, or the ring conformation is dramatically different such that the C3 protons are not able to hyperconjugate with the oxocarbenium ion as well.

A primary ^{14}C isotope effect of 1.027 ± 0.010 was measured with the isotopomeric pair of [^{3}H-NAc] and [2-^{14}C] UMP-NeuAc and lactose as the acceptor. This is identical within experimental error to the primary ^{14}C effect measured for $\alpha(2\rightarrow6)$ sialyltransferase with UMP-NeuAc and much larger than the value seen with the natural donor substrate CMP-NeuAc. This value is

in the range seen for reactions that proceed with a dissociative mechanism (101). The transition-state is similar to that seen for $\alpha(2\rightarrow6)$, but with less bond cleavage to the leaving group as shown in Figure 6-2.

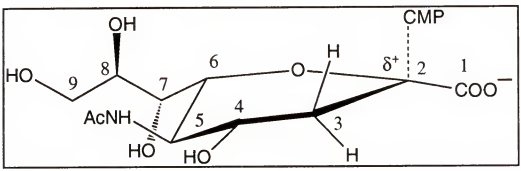


Figure 6-2. Transition-state model for $\alpha(2\rightarrow3)$ sialyltransferase.

Solvent Deuterium Isotope Effects

The pattern of solvent deuterium isotope effects measured for $\alpha(2\rightarrow3)$ sialyltransferase were very different for those measured for $\alpha(2\rightarrow6)$ sialyltransferase. With both CMP-NeuAc and UMP-NeuAc, large effects (≈2) were measured under V/K conditions, but unity effects were seen under V_{max} conditions. While the reason for this is currently unclear, it does indicate

that there are significant differences in the kinetic mechanisms for the $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ enzymes.

Conclusions

The family of isotope effects measured for the $\alpha(2\rightarrow3)$ sialyltransferase system support a transition-state that has substantial oxocarbenium ion character similar to that of $\alpha(2\rightarrow6)$ sialyltransferase. However, the are measurable differences between the β-dideuterium effects for the two enzymes indicating less charge build-up for the $\alpha(2\rightarrow3)$ sialyltransferase. This could be due to the reaction proceeding with an earlier transition-state. This difference in the transition-state structures is important because it may offer a way to make sialyltransferase inhibitors that are specific. Differences in the solvent deuterium isotope effects also suggest significant differences in the two mechanisms.

Experimental

General α(2→3) Sialyltransferase Kinetic Isotope Effect Methodology

Kinetic isotope effect (KIE) experiments on α(2→3) sialyltransferase reactions used mixtures of radiolabeled isotopomeric substrates, and were therefore competitive (112). Experiments employed ca. 100,000 CPM each of the appropriate ^{3}H- and ^{14}C- labeled CMP-NeuAc isotopomers. For each experiment a master of a given $^{3}H/^{14}C$-labeled isotopomeric pair of CMP-NeuAc containing the appropriated amount of lactose (9.4 mM) was prepared from which aliquots were withdrawn for individual reactions and measurement of the reference $^{3}H/^{14}C$ ratio at time 0. Reaction mixtures (50-500 μL, 40 mM cacodylate, 0.2 mg/mL BSA, 0.2% Triton CF-54, pH 7) were initiated by the addition of the appropriate amount of enzyme to give 40-60% conversion (68) in 30-60 minutes. After 40-60% conversion, unreacted substrate was isolated from the reaction

mixture on a MonoQ HR10/10 column (100 mM NH_4HCO_3, 15% methanol, pH 8.0, 2 mL/min, A_{260}) and collected directly into scintillation vials (2 mL fractions). Care was taken to collect the entire CMP-NeuAc peak. The percent conversion was determined from the CMP-NeuAc and CMP peaks in the HPLC chromatogram. The initial $^3H/^{14}C$ ratio was obtained in triplicate by injecting aliquots of the master mixture of labeled CMP-NeuAc on the MonoQ column and recollecting the entire CMP-NeuAc peak. The $^3H/^{14}C$ ratios for CMP-NeuAcs were determined by dual-channel liquid scintillation counting (channel A, 0-15 keV; channel B 15-80 keV) with each tube being counted for 10 min, and all tubes cycled through the counter 6-10 times. The internal ^{133}Ba source was used to estimate the quench parameter for each tube, which varied by ±1%. Triplicate samples of [^{14}C] CMP-NeuAc were used to determine the ratio of ^{14}C counts in channels A and B ($A:B_{14}$). Since ^3H is only detected in channel A, the $^3H/^{14}C$ ratio in a given tube was calculated with the following equation (112):

$^{3}H/^{14}C$= (cpm A-cpm B X $A:B_{14}$) / (cpm B + cpm B X $A:B_{14}$) eq. 6.1

The reported value and error of a KIE represents the mean and standard deviation of three individual KIE experiments taken over 6-10 cycles through the liquid scintillation counter. The following equations were used to calculate the KIEs:

$$\beta\text{-}^{2}H\ KIE_{observed} = (^{3}H/^{14}C)_{t1/2} / (^{3}H/^{14}C)_{0} \quad \text{eq. 6.2}$$

$$^{14}C\ KIE_{observed} = (^{14}C/^{3}H)_{t1/2} / (^{14}C/^{3}H)_{0} \quad \text{eq. 6.3}$$

The KIEs were then corrected for % conversion using the following equation (69):

$$KIE_{corrected} = \ln(1-f) / \ln((1-f) \times KIE_{observed}) \quad \text{eq. 6.4}$$

f = %conversion

Kinetic isotope effect experiments for the $\alpha(2\rightarrow3)$ sialyltransferase system using the slow substrate UMP-NeuAc

were also carried out in a similar manner. The differences were that substantially more enzyme (ca. 10 fold) was used to reach 40-60% conversion in the desired time frame, and the elution time from the MonoQ column for UMP-NeuAc was slightly longer than that of CMP-NeuAc. However, this presented no difficulty in the ability to collect the entire unreacted UMP-NeuAc peak. A final difference was that much greater concentrations of the acceptor substrate ([lactose] = 200 mM) were routinely used to achieve acceptable % conversions.

Isotope effect utilizing a slow acceptor substrate (galactose) for the $\alpha(2\rightarrow3)$ sialyltransferase were also performed. These were done as above with CMP-NeuAc as the donor substrate and 500 mM galactose.

Steady State Kinetics for UMP-NeuAc with $\alpha(2\rightarrow3)$ Sialyltransferase

To estimate the kinetic parameters for UMP-NeuAc, its concentration was varied while keeping LacNAc saturating. Five reaction mixtures (70 μL each) were prepared with the following

UMP-NeuAc concentrations: 550, 200, 100, 67, and 50 μM. Each reaction mixture was made 500 mM lactose, 40 mM cacodylate, 0.2 mg/mL BSA, 0.2% Triton CF-54, pH 7.5. Each reaction contained 0.014 μCi (30,000 CPM) of [^{14}C-NAc] UMP-NeuAc. The mixtures were pre-incubated at 37 °C for 1 minute. Reactions were initiated by the addition of 10-15 mU enzyme. Aliquots (26 μL) were removed at 4, 8, and 12 minutes. Product analysis utilized the Dowex column methodology.

To estimate the kinetic parameters for LacNAc, its concentration was varied while keeping UMP-NeuAc saturating. Five reaction mixtures (80 μL each) were prepared with the following lactose concentrations: 500, 200, 67, 40, and 25 mM. Each reaction mixture was made 500 μM UMP-NeuAc, 40 mM cacodylate, 0.2 mg/mL BSA, 0.2% Triton CF-54, pH 7.5. Each reaction contained 0.014 μCi (30,000 CPM) of [^{14}C-NAc] UMP-NeuAc. The mixtures were pre-incubated at 37 °C for 1 minute. Reactions were initiated by the addition of 15-25 mU enzyme. Aliquots (30 μL) were removed at 6, 12 and 18 minutes. Product analysis utilized the Dowex column methodology.

Solvent Isotope Effects with CMP-NeuAc

For effects under V_{max} conditions, a 1 mL solution was prepared consisting of 500 μM [1-^{14}C] CMP-NeuAc (0.139 mCi/mmol), 100 mM lactose, 40 mM cacodylate, 0.2 mg/mL BSA, and 0.2% Triton CF-54 (pH 6.5). Half of this mixture was rotovapped to dryness *in vacuo* and exchanged with $^{2}H_2O$ (99.9% ^{2}H; Cambridge Isotope Laboratories, Inc.) two times and brought to a final volume of 500 μL in $^{2}H_2O$. It was determined that this solution had a pD of 6.2 by measurement with a pH meter. This is equivalent to a pH of 6.6 (pH = pD + 0.4) due to the isotope effect on the pH probe (138). Reactions (200 μL) were run in duplicate in H_2O and $^{2}H_2O$. Reactions were initiated by the addition of 0.95 mU α(2→3) sialyltransferase and were run at 37 °C, with 65 μL aliquots removed at 8, 16 and 24 minutes. Product analysis utilized the Dowex column methodology.

For effects under V/K (CMP-NeuAc) conditions, a 1 mL solution was prepared consisting of [1-^{14}C] CMP-NeuAc (20 mCi/mmol), 100 mM lactose, 40 mM cacodylate, 0.2 mg/mL BSA, and 0.2% Triton CF-54 (pH 6.5). Half of this mixture was

rotovapped to dryness *in vacuo* and exchanged with ^2H_2O (99.9% ^2H; Cambridge Isotope Laboratories, Inc.) two times and brought to a final volume of 500 μL in ^2H_2O. It was determined that this solution had a pD of 6.2 by measurement with a pH meter. This is equivalent to a pH of 6.6 (pH = pD + 0.4) due to the isotope effect on the pH probe (138). Reactions (80 μL) were run in duplicate in H_2O and ^2H_2O. Reactions were initiated by the addition of 0.95 mU α(2→3) sialyltransferase and were run at 37 °C, with 25 μL aliquots removed at 8, 16 and 24 minutes. Product analysis utilized the Dowex column methodology.

Solvent Isotope Effects with UMP-NeuAc

For effects under V_{max} conditions, a 1 mL solution was prepared consisting of 2 mM [1-^{14}C] UMP-NeuAc (0.010 mCi/mmol), 100 mM lactose, 40 mM cacodylate, 0.2 mg/mL BSA, and 0.2% Triton CF-54 (pH 6.5). Half of this mixture was rotovapped to dryness *in vacuo* and exchanged with ^2H_2O (99.9% ^2H; Cambridge Isotope Laboratories, Inc.) two times and brought to a

final volume of 500 μL in ^2H_2O. It was determined that this solution had a pD of 6.2 by measurement with a pH meter. This is equivalent to a pH of 6.6 (pH = pD + 0.4) due to the isotope effect on the pH probe (138). Reactions (200 μL) were run in duplicate in H_2O and ^2H_2O. Reactions were initiated by the addition of 10 mU α(2→3) sialyltransferase and were run at 37 °C, with 65 μL aliquots removed at 8, 16 and 24 minutes. Product analysis utilized the Dowex column methodology.

For effects under V/K (CMP-NeuAc) conditions, a 1 mL solution was prepared consisting of [1-^{14}C] UMP-NeuAc (20 mCi/mmol), 100 mM lactose, 40 mM cacodylate, 0.2 mg/mL BSA, and 0.2% Triton CF-54 (pH 6.5). Half of this mixture was rotovapped to dryness *in vacuo* and exchanged with ^2H_2O (99.9% ^2H; Cambridge Isotope Laboratories, Inc.) two times and brought to a final volume of 500 μL in ^2H_2O. It was determined that this solution had a pD of 6.2 by measurement with a pH meter. This is equivalent to a pH of 6.6 (pH = pD + 0.4) due to the isotope effect on the pH probe (138). Reactions (80 μL) were run in duplicate in H_2O and ^2H_2O. Reactions were initiated by the addition of 8 mU

$\alpha(2\rightarrow3)$ sialyltransferase and were run at 37 °C, with 25 μL aliquots removed at 8, 16 and 24 minutes. Product analysis utilized the Dowex column methodology.

CHAPTER 7
CONCLUSIONS AND FUTURE WORK

Conclusions

This work has resulted in a better understanding of the mechanism and transition-state structure of the reactions catalyzed by the $\alpha(2\rightarrow6)$ and $\alpha(2\rightarrow3)$ sialyltransferases. Additionally, information has been obtained pertaining to the chemical reactivity of the donor substrate CMP-NeuAc. The synthesis and utility of an alternate substrate, UMP-NeuAc, for sialyltransferases has also been demonstrated.

Through the use of kinetic isotope effects, it was shown that the acid catalyzed hydrolysis of CMP-NeuAc proceeds via a dissociative type mechanism with substantial oxocarbenium ion character in the transition-state. These results in conjunction with trapping experiments in the presence of methanol suggest that this reaction proceeds without nucleophilic assistance from the carboxylate group of CMP-NeuAc, or solvent.

The isotope effects on $\alpha(2\rightarrow6)$ sialyltransferase with the natural donor substrate CMP-NeuAc were unusual and indicated

that there was a significant commitment to catalysis associated with the reaction. This prompted the synthesis of a slow substrate, UMP-NeuAc, which allowed the intrinsic isotope effects on this system to be measured. The intrinsic isotope effects suggest that the transition-state for the enzyme catalyzed reaction has substantial oxocarbenium ion character and is very similar to that for the acid catalyzed solvolysis of CMP-NeuAc. This transition-state is similar to that proposed for fucosyltransferase, and may prove to be general for many, if not all, glycosyltransferases. The measurement of intrinsic isotope effects with UMP-NeuAc, as well as the disappearance of the solvent deuterium isotope effects when using UMP-NeuAc, suggest that there is a step in the kinetic mechanism preceding glycosidic bond cleavage. This step may be a conformational change and could require the presence of the second substrate to "trigger" it. The change in the observed pH-rate curves in going from CMP-NeuAc to UMP-NeuAc support a change in the rate-determing step, while the curve for UMP-NeuAc is consistent with a general acid and general base mechanism.

The kinetic isotope effects observed for $\alpha(2\rightarrow3)$ sialyltransferase are not drastically different than those for $\alpha(2\rightarrow6)$ sialyltransferase, but are different enough to suggest that their are important differences between the two transition-states. Additionally, the effects measured for the $\alpha(2\rightarrow3)$ enzyme are much different than those observed for solvolysis of CMP-NeuAc, suggesting that the $\alpha(2\rightarrow3)$ reaction proceeds with a much earlier transition-state than solvolysis.

The work completed in this project has implications on inhibitor design. Our results point towards a transition-state for sialyltransferases which possess substantial positive charge, and this characteristic should be incorporated into possible transition-state analogs. The recent work by Schmidt clearly shows the importance of the cytidine moiety in binding, as well. Therefore, combining a CMP group to structures which possess a positive charge at the linkage position have the potential to be bound very tightly by the enzyme. A proposed transition-state analog is shown below in Figure 7-1. The ring portion of the molecule was taken from sialidase inhibitors synthesized by Dr.

Ian Parr in our lab and possesses a positive charge which could mimic the oxocarbenium ion character of the sialyltransferase transition-state (140). The R group in the sialidase inhibitors was either H or a phenyl group, however, the work by Schmidt indicates that a phosphonate group at this position would add favorable binding interactions.

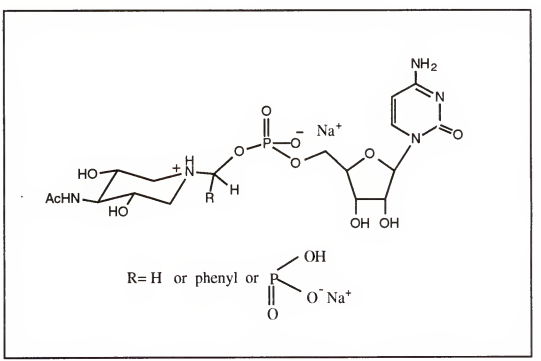


Figure 7-1. Proposed sialyltransferase transition-state analog.

Future Work

The work completed using UMP-NeuAc as an alternate substrate for sialyltransferases has opened up many new avenues for exploration of these enzymes. Because all members of the sialyltransferase family utilize CMP-NeuAc as the donor substrate, UMP-NeuAc should be a viable slow substrate for the other enzymes in this family as well. The immediate future of this work should see careful examination of $\alpha(2\rightarrow3)$ sialyltransferase with UMP-NeuAc in order to allow for more comparisons to be made with $\alpha(2\rightarrow6)$ sialyltransferase. This work could include pH-rate profiles as well as the measurement of additional isotope effects such as leaving group effects or effects on the incoming nucleophile. Later work could extend the use of UMP-NeuAc to other sialyltransferases.

Continuing work on the $\alpha(2\rightarrow6)$ sialyltransferase system will most likely begin with positional isotope exchange (PIX) experiments on the enzyme. If and where protonation of the leaving group phosphate occurs is an area that deserves attention. Clearly protonation of the bridging oxygen would be most

advantageous with respect to bond cleavage, however, the non-bridging oxygen carrying the negative charge is much more basic. How the enzyme deals with this in catalysis is an interesting question.

An experiment which would be very informative is the measurement of an equilibrium binding isotope effect at the C9 position of NeuAc. This experiment would require an apparatus with two chambers separated by a membrane which has a molecular weight cutoff below that of sialyltransferase (141). One would be filled with enzyme and substrate while the other would only contain substrate. The substrate would be a mixture of two isotopomers with trace labels to allow the effect to be determined. After equilibration, the isotope effect would be determined be the relative ratio of substrates in the two chambers (141). Clearly there will be many experimental challenges in such an experiment given the instability of CMP-NeuAc, but the information available from such an experiment would make it a worthwhile endeavor. This experiment would indicate whether the binding effect at this position was

occurring during the formation of the Michaelis complex, or after its formation and during catalysis. If an equilibrium binding effect is observed in this experiment, it would indicate that this effect is occurring in the formation of the Michaelis complex.

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BIOGRAPHICAL SKETCH

Michael John Bruner, son of John W. and Lee B. Bruner, was born in Melbourne, Florida, on February 23, 1972. Shortly afterward he was relocated to Shalimar, Florida, where he completed his early education culminating in graduation from Choctawhatchee High School, Ft. Walton Beach, Florida, in 1990. He attended the University of West Florida, where he graduated with a bachelor of science degree in chemistry in 1994 and received the Chemistry Student of the Year Award his senior year. He received his doctor of philosophy degree in chemistry from the University of Florida in summer of 1999 under the guidance of Dr. Benjamin A. Horenstein. He was awarded an American Society for Microbiology/National Center for Infectious Diseases postdoctoral fellowship at the Centers for Disease Control in Atlanta, Georgia, where he will begin a two-year stay beginning in the summer of 1999.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of
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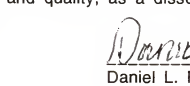
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Merle Battiste
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1999

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Dean, Graduate School